SOIL MICROBIAL RESPONSE TO GLYPHOSATE-BASED COTTON PEST MANAGEMENT SYSTEMS

A Dissertation

by

SARAH RENEE LANCASTER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Agronomy

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Approved by:

Chair of Committee,	Scott A. Senseman
Committee Members,	James M. Chandler
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ABSTRACT

Soil Microbial Response to Glyphosate-based Cotton Pest Management Systems.

(May 2008)

Sarah Renee Lancaster, B.S., University of Missouri;

M.S., North Carolina State University

Chair of Advisory Committee: Dr. Scott A. Senseman

Currently, 74% of cotton acres in the United States are planted with glyphosate-tolerant varieties. The average glyphosate-tolerant cotton crop is treated with glyphosate 2.1 times each year in addition to other herbicides, insecticides, and fungicides. The primary objectives of this research were to: 1) describe the influence of glyphosate and pesticides commonly applied at or near the time of cotton planting on soil microbial activity and biomass; 2) study the effect of glyphosate on fluometuron degradation; 3) evaluate the response of *Rhizoctonia solani* to glyphosate and fluometuron; 4) study changes in glyphosate metabolism that occur as a result of repeated glyphosate applications; and 5) define shifts in the soil microbial community. Additionally, methods for accelerated solvent extraction (ASE) of fluometuron from soils were developed.

In one experiment, the addition of glyphosate reduced C-mineralization in soils treated with fluometuron, aldicarb, or mefenoxam + PCNB formulations. However, in a second

experiment, C-mineralization increased when glyphosate was applied with fluometuron relative to fluometuron applied alone.

Accelerated solvent extraction was used in experiments which demonstrated that application of glyphosate with fluometuron increased the rate of fluometuron degradation in soil relative to fluometuron alone. When glyphosate was added to minimal medium, degradation of fluometuron by *R. solani* was reduced and less fungal biomass was produced. The total amount of ^{14}C-glyphosate mineralized was reduced when glyphosate was applied 5 times relative to 1, 2, 3, or 4 times. Incorporation of ^{14}C-glyphosate residues into soil microbial biomass was greater following five glyphosate applications than one application 3 and 7 days after application (DAA). Soil fatty acid methyl ester (FAME) profiles were altered by five glyphosate applications relative to one application. Additionally, FAMEs common to gram-negative bacteria were present in higher concentrations following five applications relative to 1, 2, 3, or 4 applications both 7 and 14 DAA.

These studies indicated that: 1) glyphosate altered the soil microbial response to other pesticides; 2) fluometuron-degrading microorganisms in soil responded differently to glyphosate; 3) changes in the dissipation or distribution of glyphosate following repeated glyphosate applications were associated with changes in the structural diversity of the soil microbial community.

DEDICATION

To my husband, Phillip, who always believes in me.

And to my grandparents, who taught me to value kindness and perseverance.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Scott Senseman, for his valuable guidance and support throughout my time at TAMU. I would like to express my appreciation for my committee members, Drs. Mike Chandler, Frank Hons, and Charles Kenerley for their assistance. I also thank Drs. Rick Haney and Terry Gentry for their generous contributions of time and materials. Special thanks go to Dr. Kathy Carson, not only for her help with my research, but also for her support and friendship. Thanks also go to my fellow graduate students, especially Luis Avila, Mark Matocha, Edinalvo Camargo, Sam Willingham, Greg Steele, Nyland Falkenberg, Josh Bynum, and Polly Longenberger for their help and companionship. Finally, thanks to my husband for his constant encouragement and support.

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CHAPTER I

INTRODUCTION

Cotton was produced on approximately 12.4 million acres in the United States in 2005 (*1*). Cotton production typically requires intensive chemical management of pests including weeds (*2*), insects (*3*), and diseases (*4*). However, cotton varieties have recently been genetically engineered to reduce reliance on pesticides that pose large environmental or health risks, specifically herbicides (*5*) and insecticides (*6*). The introduction of glyphosate-tolerant crops in 1996 (*7*) has had a tremendous impact on weed management. Currently, 74% of cotton planted in the United States is glyphosate-tolerant varieties (*1*). The average glyphosate-tolerant cotton crop is treated with glyphosate 2.1 times each year (*1*). However, glyphosate may be applied up to four times during the cotton growing season (*8*). In addition, glyphosate may be applied prior to planting or used as a harvest aid.

The popularity of glyphosate-tolerant varieties has been driven by improved weed control (*7*), low herbicide cost (*5*), ease of application (*7*), and low environmental and toxicological risk from the herbicide (*9*). From an environmental perspective, the use of glyphosate would be preferred over other chemistries that exhibit greater soil mobility, half-life, or toxicity. However, glyphosate-based weed control systems have led to

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extensive use of and dependence on the herbicide.

It is reasonable to believe that glyphosate would increase microbial metabolism because of the herbicide's low C:N ratio of 3:1 (*10*). Additionally, there are some bacteria that utilize glyphosate as a source of P (*11*). It has been reported that glyphosate is rapidly degraded by soil microbes, presumably by co-metabolism (*12*) and results in increased microbial biomass and activity (*10, 13*).

When glyphosate application coincides with the application of soil-active pesticides, it is possible that their behavior in the soil may be modified, particularly with regard to microbial degradation. For example, when glyphosate and atrazine were applied simultaneously to treated soils, microbial activity was generally enhanced; however, atrazine degradation varied, possibly due to differences in herbicide concentration (*14, 15*). Atrazine degradation was reduced by the presence of glyphosate 8 and 12 days (d) after herbicide application (*14*). However, after 14 to 28 d atrazine degradation was enhanced when glyphosate was also present in the soil (*15*). The authors' attribute this altered degradation to changes in the soil microbial population.

In a cotton production system, there is potential for other pesticides to be present in the soil profile at the time of a glyphosate application. These include the herbicides trifluralin or fluometuron, the fungicides PCNB and mefenoxam, and the insecticide/nematicide aldicarb. These products are metabolized, to varying degrees, by soil microbes (*16, 17, 18*) and may alter the activity of the soil microbial community.

Repeated applications of one pesticide may have a greater impact on the soil microflora than a single application. For example, it has been reported that repeated

applications of pendimethalin over five years led to enhanced degradation resulting in 15% less pendimethalin present in soil after ten repeat applications compared to soil that had only one application of pendimethalin (*19*). Additionally, repeated applications of aldicarb over 20 years enhanced degradation of aldicarb metabolites (*20*). Alternatively, scientists in Brazil reported lower carbon (C) mineralization and extended glyphosate half-life following two, three, or four applications of glyphosate relative to one application (*21*).

Changes in microbial activity may indicate a response of the microbial community structure as it adapts to management practices. Ka et al. (*22*) used fatty acid methyl ester (FAME) profiles to study soil microbial communities from soils treated with the herbicide 2,4-D. They were able to relate FAME profiles with both genetic and functional characteristics. It is reasonable to believe, therefore, that potential changes in soil function resulting from glyphosate applications may be better understood by studying shifts in the community structure as described by FAMEs.

Altered pesticide degradation is just one possible outcome of changes in the soil microbial community that result from pesticide applications. The selection pressure applied on the soil microbial community when pesticides are applied may disrupt the balance of the microbial community and result in more soil bacteria or fungi that have pathogenic properties. For example, Descalzo et al. (*23*) demonstrated that glyphosate applications to common bean and sunflower resulted in an increased *Pythium* population.

Pathogenic fungi may also have important roles in the degradation of pesticides. For example, *Rhizoctonia solani* is one organism that degrades phenylurea herbicides such as fluometuron (*24*, *25*). It is unknown how this organism responds to glyphosate in a soil environment.

Given the limited knowledge concerning the details of soil microbial communities, it is difficult to predict the impact of pesticides as a selection pressure on all their functions. Nonetheless, scientific evidence suggests that anthropogenic activities influence genetic diversity and the potential that a soil microbial community possesses.

The goals of this research are to: 1) describe the influence of glyphosate and pesticides commonly applied at or near the time of cotton planting on soil microbial activity and biomass; 2) study the effect of glyphosate on fluometuron degradation; 3) evaluate the response of *Rhizoctonia solani* to glyphosate and fluometuron; and 4) define shifts in glyphosate metabolism and soil microbial community structure that occur as a result of repeated glyphosate applications.

CHAPTER II

SOIL MICROBIAL ACTIVITY IS AFFECTED BY ROUNDUP WEATHERMAX® AND PESTICIDES APPLIED TO COTTON (*Gossypium hirsutum*)*

INTRODUCTION

Cotton production typically requires intensive chemical management of weeds (*2*), insects (*3*), and diseases (*4*). However, varieties of cotton and other crops have recently been genetically engineered to reduce reliance on pesticides that pose environmental or health risks, specifically herbicides (*5*) and insecticides (*6*). The introduction of glyphosate-tolerant crops in 1996 (*7*) had a tremendous impact on weed management (*26*). From an environmental perspective, the use of glyphosate {*N*-(phosphonomethyl)glycine} would be preferred over other herbicides that exhibit greater soil mobility, half-life, or mammalian toxicity. However, glyphosate-based weed control systems have resulted in extensive use of the herbicide, with multiple applications in a single growing season becoming common (*27*).

Glyphosate may increase microbial activity because the herbicide's low carbon:nitrogen ratio represents a desirable balance of those nutrients for soil microbes (*10*). Glyphosate is rapidly degraded by soil microbes, presumably by co-metabolism (*9,*

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*Reproduced with permission from Lancaster, S.H.; Haney, R.L.; Senseman, S.A.; Hons, F.M.; Chandler, J.M. *J. Agric. Food Chem.* **2006**, 54, 7221-7226.

12), resulting in increased microbial biomass and activity (*10, 13*). Additionally, glyphosate application coincidental to soil-active pesticides may modify pesticide behavior, particularly with regard to microbial degradation. For example, when glyphosate and atrazine {6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine} were applied simultaneously to soil samples, microbial activity was generally enhanced; although atrazine degradation rates varied (*14*). Atrazine degradation was reduced by the presence of glyphosate 8 and 12 d after herbicide application (*14*). However, after 14 to 28 d, atrazine degradation was enhanced when glyphosate was present in the soil (*14*).

In cotton production systems, the herbicides trifluralin (2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethyl)benzenamine) or fluometuron [*N*,*N*-dimethyl-*N*'-{3-(trifluoromethyl)phenyl}urea], the fungicides PCNB (pentachloronitrobenzene), and mefenoxam {methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-D-alaninate}, and the insecticide-nematicide aldicarb [2-methyl-2-(methylthio)propanal *O*-{(methylamino)carbonyl}oxime] may be present in the soil at the time of glyphosate application. These products are metabolized, to varying degrees, by soil microbes (*16, 17, 18*) and may alter the activity of the soil microbial community. The effect of a commercially available glyphosate formulation on the response of carbon and nitrogen mineralization and soil microbial biomass to trifluralin, fluometuron, PCNB, mefenoxam, and aldicarb was evaluated.

MATERIALS AND METHODS

Soil. The soil used in this study was a Weswood clay loam (fine-silty, mixed superactive, thermic Udifluventic Haplustept). Bulk soil was collected from a fallow field that was previously planted to cotton and from a bermudagrass pasture. Soil characteristics are presented in **Table 1**. Soils were air-dried and passed through a 2-mm sieve prior to the beginning of the experiment.

Chemicals. Commercial formulations of glyphosate (Roundup WeatherMAX, Monsanto Company, St. Louis, MO), trifluralin (Treflan HFP, Dow AgroSciences, Indianapolis, IN), fluometuron (Cotoran 4L, Griffin L.L.C., Valdosta, GA), PCNB+mefenoxam (Ridomil Gold PC GR, Syngenta Crop Protection, Inc., Greensboro, NC), and aldicarb (Temik brand 15G, Bayer CropScience, Research Triangle Park, NC) were used (Table 2). Pesticides will be referred to by their common names throughout this paper. Reagents, except HCl, were obtained from Fisher Scientific (Fair Lawn, NJ). Hydrochloric acid was obtained from LabChem, Inc. (Pittsburg, PA) (**Table 2**).

Sample preparation. Fifty-five g of dry weight equivalent soil were placed in each gas-tight container containing 1 M KOH to trap evolved CO_2, and soils were rewetted to approximately 50% water-filled pore space (20% gravimetric water content). Samples were incubated in the dark at 30 C for 7 d to allow stabilization of the soil microbial biomass following the initial flush of activity after re-wetting (*28*). Potassium hydroxide traps were then removed and pesticide treatments were applied. Pesticides were applied in water to bring the final gravimetric water content to 25%. Rates of application were

Table 1. Selected properties of soils used in the study[a].

Soil[a]	Clay (%)	pH	O.M. (%)	NO_3	P
				———μg mL^{-1}———	
pasture	34	7.6	6.2	4	52
cultivated field	32	8.0	1.4	20	38

[a]Soil was Weswood clay loam collected from a bermudagrass pasture and a fallow field previously planted to cotton.

Table 2. Solubility, half-life, organic carbon partitioning coefficient, and chemical structure of pesticides used in the study[a].

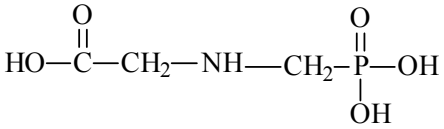
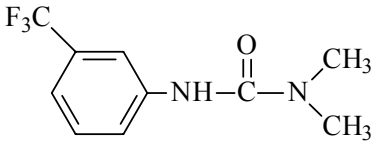
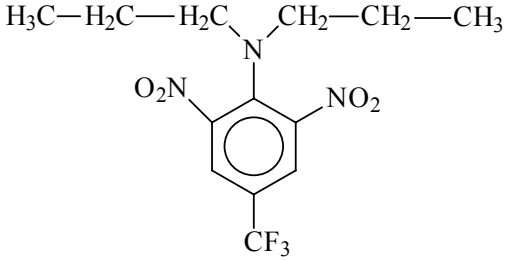
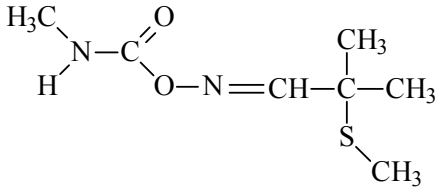
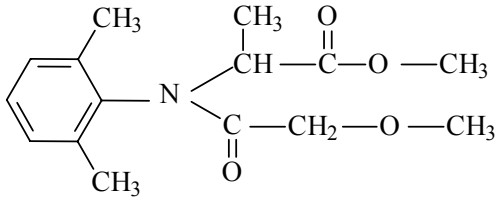
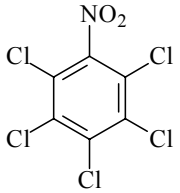
Active ingredient	S_w[b] g/L	$T_{1/2}$[b] (d)	Log K_{ow}[b]	Structure
glyphosate	12.0	3 to 174	-3.40	
fluometuron	0.08	10 to 100	2.38	
trifluralin	0.004	57 to 126	4.83	

Table 2, continued.

Active ingredient	S_w[b]	$T_{1/2}$[b]	Log K_{ow}[b]	Structure
	g/L	(d)		
aldicarb	6.0	20 to 361	0.053	
mefenoxam	7.1	19 to 730	1.75	
pentacholoronitrobenzene	0.0001	120 to 300	5.10	

[a]Information adapted from references 49 and 50.

[b]Abbreviations: S_w, water solubility; $T_{1/2}$, half-life; K_{ow}, octanol:water partioning coefficient; N/A, not available.

Table 3. Pesticide treatments applied to pasture and cultivated field soil[a].

Pesticide[b]	Interaction depth (mm)	Concentration[c, d] (μg active ingredient/kg soil)
fluometuron	37.5	4.1
trifluralin	37.5	2.0
aldicarb	37.5	10.2
mefenoxam + PCNB	37.5	8.3 + 0.42
glyphosate	1.5	152.7
glyphosate + fluometuron	1.5 + 37.5	152.7 + 4.1
glyphosate + trifluralin	1.5 + 37.5	152.7 + 2.0
glyphosate + aldicarb	1.5 + 37.5	152.7 + 10.2
glyphosate + mefenoxam + PCNB	1.5 + 37.5	152.7 + 8.3 + 0.42
untreated	--	0

[a]Soil was Weswood clay loam collected from a bermudagrass pasture and a fallow field previously planted to cotton.

[b]Pesticides were applied as formulated products: glyphosate, Roundup WeatherMax; fluometuron, Cotoran 4L; trifluralin, Treflan HFP; aldicarb, Temik 15G; and mefenoxam + pentachloronitrobenzene (PCNB), Ridomil Gold PC GR.

[c]Rate of application of formulated product: Cotoran 4L, 4.67 L/ha; Treflan HFP, 2.34 L/ha; Temik 15G, 5.62 kg/ha; Ridomil Gold OC GR, 11.23 kg/ha; and Roundup WeatherMax, 7.01 L/ha.

[d]Calculations of concentration assume mass of 15-cm furrow slice is 2,200,000 kg.

consistent with recommended rates and adjusted for effective soil interaction depths of 1.5 mm for glyphosate (*15*) and 37.5 mm for fluometuron (*29*), trifluralin (*29*), aldicarb (*16*), and mefenoxam + PCNB (*30*). Factors included two soils, two glyphosate rates and five pesticide treatments. Pesticide treatments and rates are listed in **Table 3**. Treatments were replicated three times in a split-plot experimental design with soil as the main plot and pesticide treatments as the sub-plot.

Microbial activity. Microbial activity was determined by measuring C and N mineralization (*31*). Potassium hydroxide traps were replaced at 1, 2, 3, 4, 10, and 30 days after treatment to determine the amount of CO_2 evolved during that period. The amount of CO_2 absorbed was determined by titrating the remaining base with 1 N HCl and C mineralized was calculated as described by Zibilske (*31*). Nitrogen mineralization was determined by comparing inorganic N (NO_3 and NH_4) concentration in 10 g soil sub-samples at 2, 10, and 30 days after treatment to the initial inorganic N concentration. Nitrogen analysis was completed using a Rapid Flow Analyzer (OI Analytical, College Station, TX).

Microbial biomass. Soil microbial biomass C and N were determined using the fumigation-incubation method (*32*). After 10 d of incubation, soil sub-samples were fumigated with chloroform ($CHCl_3$) and incubated in the dark at room temperature overnight. Soil samples were then placed in air-tight containers with 10 mL KOH and incubated at 30 C for an additional 10 d, after which C and N mineralized were determined as previously described. The amounts of mineralized C and N were divided by a *k* factor of 0.41, representing the fraction of biomass mineralized (*33, 34*).

Data analysis. All data were analyzed using Statistical Analysis Systems version 9.1 (SAS Institute, Inc., Cary, NC). The influence of glyphosate on daily rates of C and N mineralization was analyzed in the mixed model with replicate as a random effect and all other effects fixed. Pair-wise contrasts of each pesticide with and without glyphosate were evaluated. Cumulative C and N mineralized were analyzed using the general linear model ($\alpha = 0.10$) to compare the slopes of pair-wise contrasts of each pesticide treatment with or without glyphosate. Total C and N mineralized as well as soil microbial biomass C and soil microbial biomass N were subjected to analysis of variance using the mixed model ($\alpha = 0.10$).

RESULTS AND DISCUSSION

For all variables measured, pasture soil exhibited greater microbial activity and biomass than cultivated soil, likely due to differences in soil disturbance and management. However, soil characteristics did not interact with other factors. Therefore, data were combined across soils. Where an interaction of glyphosate with other pesticides occurred, data are presented by treatment. Other data are presented by the appropriate main effects.

Carbon and nitrogen mineralization. Rates of CO_2 accumulation for each pesticide applied with and without glyphosate are shown in **Figure 1**. Significant model terms used for slope analysis of contrasts included day, day*treatment, day*soil, soil, and day*treatment*soil. Soils treated with glyphosate exhibited an enhanced rate of C mineralization relative to untreated soils (**Figure 1A**). Other researchers have reported

enhanced C mineralization following application of both analytical grade (*35*) and formulated (*10*) glyphosate. Cumulative C mineralization after 30 d was greater in soils treated only with glyphosate compared to all other treatments. However, cumulative C mineralized in the remaining treatments was similar to untreated soils.

When fluometuron was applied in combination with glyphosate, mineralized C accumulated at a slower rate relative to when fluometuron was applied alone (**Figure 1B**). This is contradictory to data reported by Bozarth and Funderburk (*36*) showing that the addition of glucose enhanced CO_2 evolution in soils treated with analytical grade fluometuron. It was expected that the addition of glyphosate should have served as a source of nutrients, thereby enhancing microbial growth and pesticide degradation. It should be noted, however, that glyphosate was applied as the formulated product in this experiment and there is evidence of microbial suppression caused by surfactants in the formulation (*13*). Furthermore, other researchers concluded that complete degradation of phenylurea herbicides, such as fluometuron, requires multiple, inter-dependent bacterial species (*37*). It is possible that fluometuron was toxic to the microorganisms that were the primary metabolizing agents of glyphosate, yet allowed fluometuron-metabolizing microorganisms to function, albeit at a slower rate than microorganisms metabolizing glyphosate applied alone.

The rate of C mineralization in soils treated with trifluralin was not influenced by the addition of glyphosate (**Figure 1C**). Trifluralin is a dinitroanaline herbicide that inhibits mitosis in susceptible species (*38*) and is susceptible to biodegradation (*39*). Previous

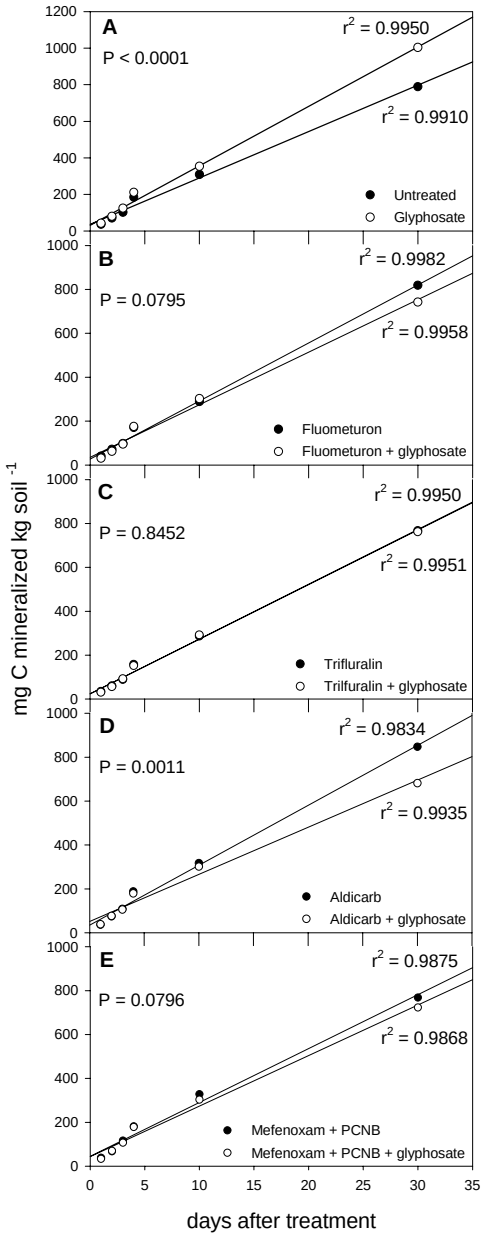


Figure 1. Effect of glyphosate on cumulative C mineralization in soil between 1 and 30 days after treatment. P values indicate significance of test for different slopes (α=0.10). (A) no herbicide and glyphosate; (B) fluometuron and fluometuron + glyphosate; (C) trifluralin and trifluralin + glyphosate; (D) aldicarb and aldicarb + glyphosate; and (E) mefenoxam + pentachloronitrobenzene (PCNB) and mefenoxam + PCNB + glyphosate.

research (*40, 41*) indicates that formulations of pendimethalin, another dinitroanaline herbicide, have negative effects on the growth of fungal species. It is likely that the herbicide would negatively impact the activity of glyphosate-metabolizing microorganisms, thereby reducing their activity.

Carbon was mineralized at a slower rate in soils that were treated with glyphosate and aldicarb relative to soils treated only with aldicarb (**Figure 1D**). Jones and Norris (*42*) reported that microbially-mediated oxidation of aldicarb begins immediately upon application to soil. Other reports (*43*) indicate that repeated application of formulated aldicarb results in increased bacterial populations with decreased diversity. Increases in one segment of the bacterial population at the expense of species diversity may reduce biodegradation of compounds such as glyphosate that are not utilized by the enhanced group of bacterial microorganisms.

Accumulation of mineralized C in soils treated with mefenoxam + PCNB decreased in response to glyphosate addition (**Figure 1E**). It has been reported that a mefenoxam-based fungicide increased the bacterial population of soil (*44*). Furthermore, Lièvremont et al. (*45*) suggested that metabolism of analytical grade PCNB by unaffected fungal species may be greater under C-limited conditions. This may account for greater microbial activity in soils treated with mefenoxam + PCNB alone. The reduction in microbial activity in soils treated with mefenoxam + PCNB and glyphosate relative to soils treated with glyphosate alone is likely due to the microbial selection caused by these pesticides.

Cumulative N mineralization 30 days after treatment is shown in **Figure 2**. Nitrogen mineralization was greater in soils that had been treated with applications that included glyphosate compared with soils that were not treated with glyphosate. This result is in agreement with Haney et al. (*13*), who reported enhanced N mineralization following the addition of glyphosate in eight of nine soils evaluated. No differences in N mineralization rates were observed when pair-wise contrasts were evaluated (data not shown).

Cumulative C and N mineralized were well correlated in treatments containing glyphosate, but were not in treatments that did not contain glyphosate (**Figure 3)**. This result indicated the strong influence of glyphosate on microbial activity, possibly due to the availability of C and N in the herbicide. Haney et al. (*13*) reported an increase in C and N mineralization equal to theamounts of C and N added as glyphosate when several soils were studied. The increased mineralization observed in these studies was not of equal magnitude; however, the addition of other pesticides is likely to have altered activity of the soil microfauna.

Soil microbial biomass. The addition of glyphosate did not affect the response of soil microbial biomass C to any pesticide. However, soil microbial biomass C increased relative to non-treated soils when glyphosate was applied alone (**Figure 4**). Haney et al. (*13*) found that soil microbial biomass C increased due to the addition of Roundup Ultra in five of nine soils evaluated. Soil microbial biomass N was not affected by any pesticide treatment (**Figure 5**). This is similar to results following the application of Roundup Ultra reported by Haney et al. (*10*), who hypothesized that soil microbial

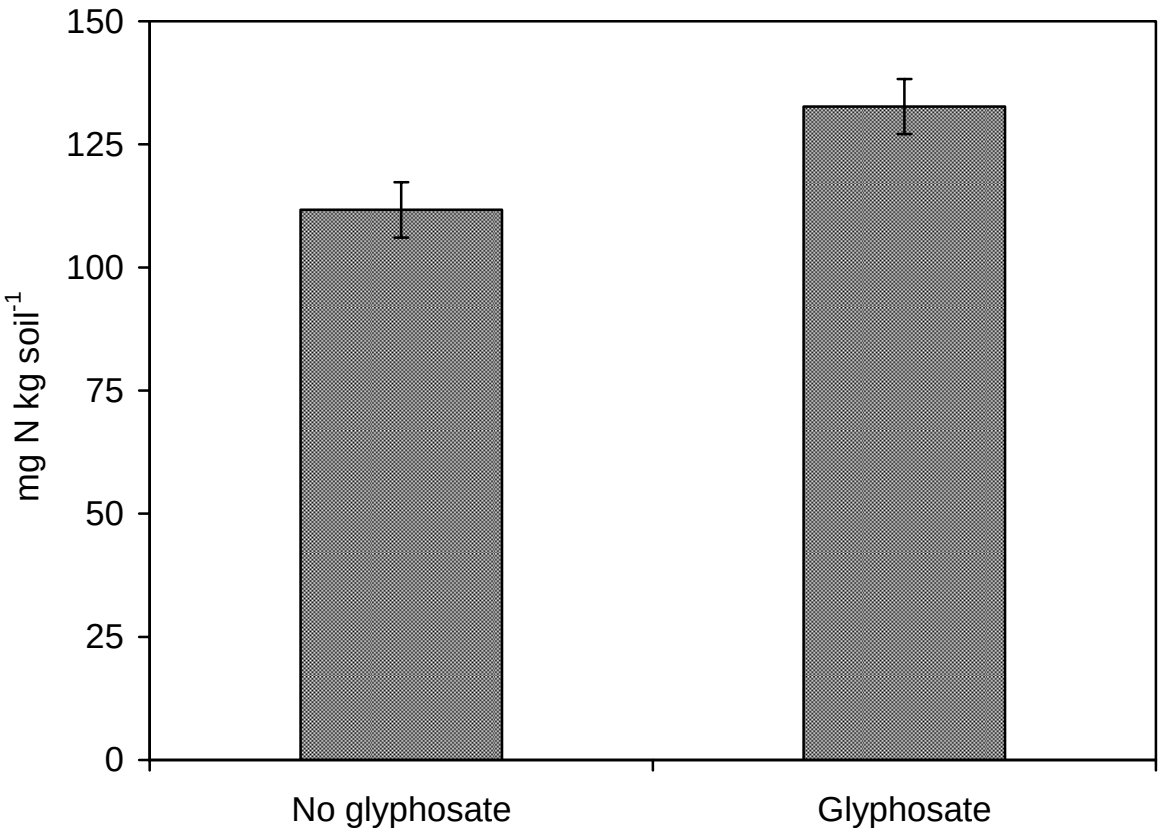


Figure 2. Nitrogen mineralized during 30 days of incubation in all pesticide treatments with and without glyphosate. Bars represent least significant difference (α=0.10) of 5.6 (P = 0.0114).

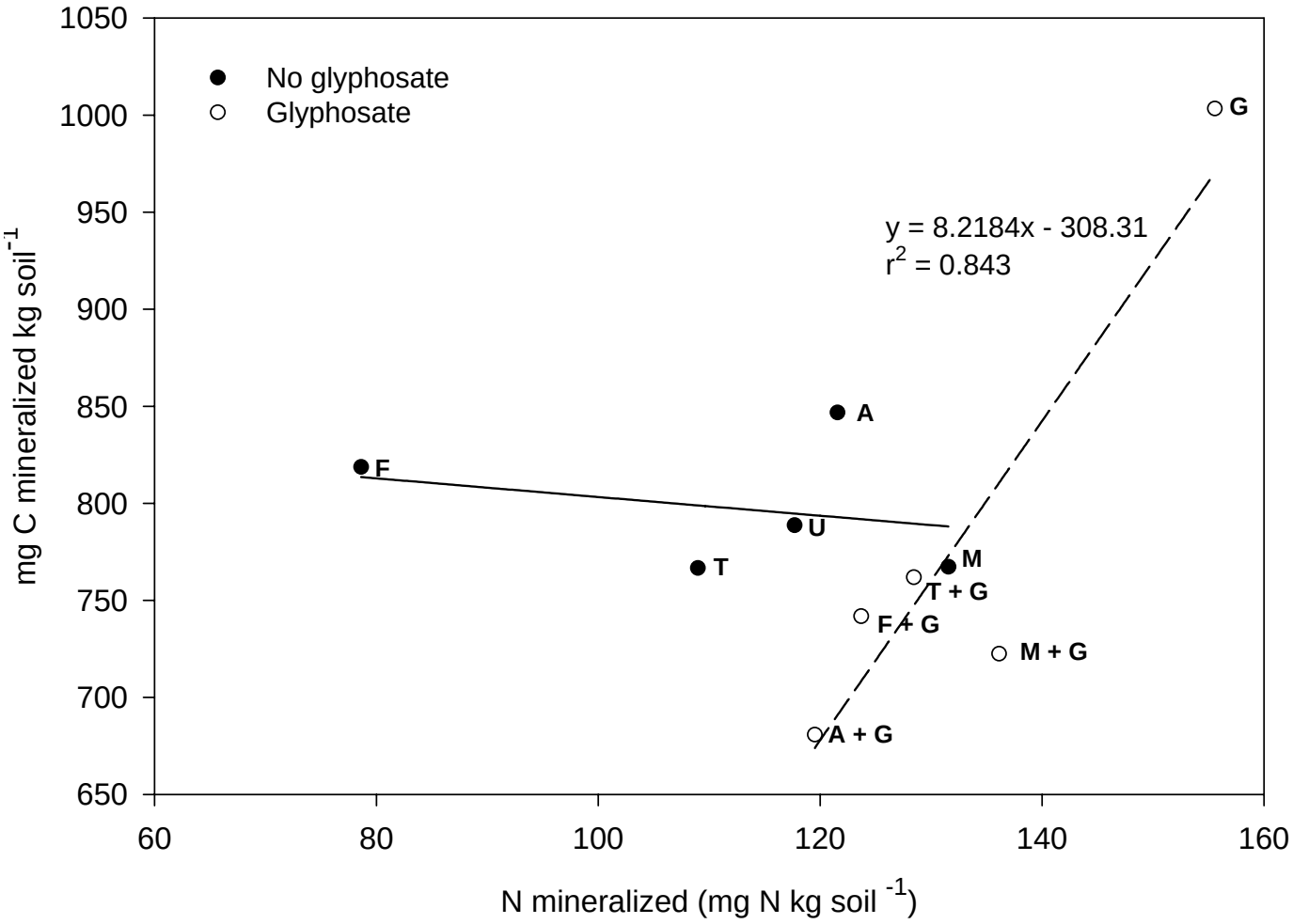


Figure 3. Cumulative C and N mineralized in 30 d after application of pesticides (A, aldicarb; F, fluometuron; M, mefenoxam + pentachloronitrobenzene (PCNB); T, trifluralin; U, untreated) alone or with glyphosate (G).

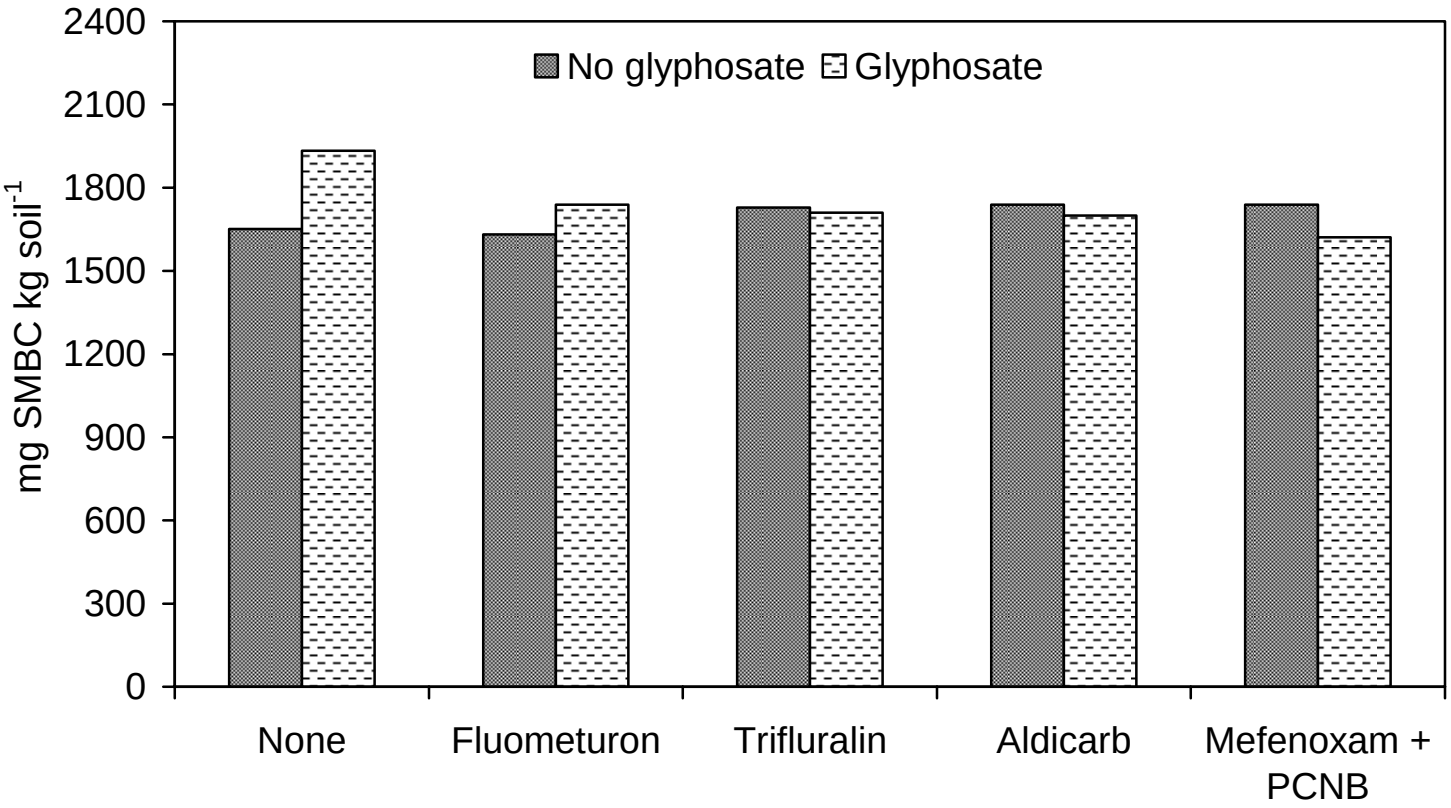


Figure 4. Soil microbial biomass C as influenced by pesticide treatments. Standard error 107.7 (P = 0.0983).

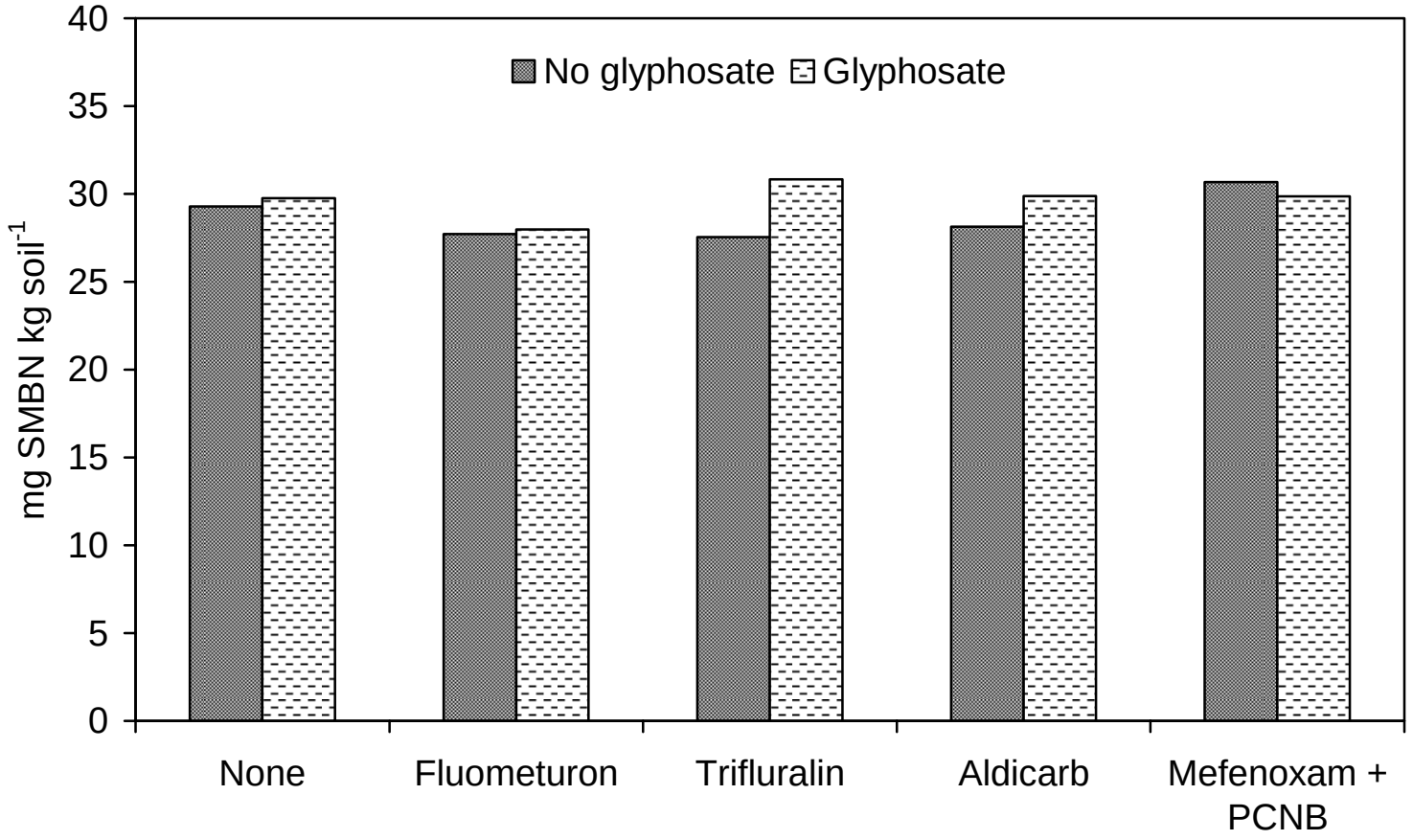


Figure 5. Soil microbial biomass N (SMBN) as influenced by pesticide treatments. Standard error 2.1 (P = 0.672).

biomass measurements using the fumigation-incubation method are less sensitive than C and N mineralization measurements for detecting the influence of microbial activity (*10*). Slow-growing bacteria have been implicated in glyphosate metabolism (*46*), preventing potential changes in soil microbial biomass from being observed during the incubation time used in these studies.

The data presented herein suggest that the response of the soil microbial community is altered when glyphosate application coincides with the presence of fluometuron, aldicarb, or mefenoxam + PCNB in the soil relative to when these pesticides are applied singly. This is not surprising given that glyphosate (*9, 12*) and many other pesticides (*47*) are degraded via co-metabolism, suggesting that the presence of other energy sources will influence the activity of the microbial community. Glyphosate is a desirable substrate for soil microfauna because, in addition to C and N, it also contains phosphorus which may result in enhanced degradation due to microbial requirements (*48*). Conversely, the presence of adjuvants in commercial formulations may adversely affect the microbial community (*13*). Additional research is needed to elucidate the effects of pesticide combinations on pesticide degradation and soil microbial community structure and function.

CHAPTER III

MICROBIAL DEGRADATION OF FLUOMETURON IS INFLUENCED BY ROUNDUP WEATHERMAX®*

INTRODUCTION

The introduction of glyphosate-tolerant crops in 1996 (*7*) has substantially impacted herbicide use (*26*). The popularity of glyphosate-tolerant varieties has been driven by broad-spectrum weed control (*7*), low herbicide cost (*5*), ease of application (*7*), and low environmental and toxicological risk of the herbicide (*9*). However, glyphosate-based weed management systems have resulted in extensive use of the herbicide. In 2006, 91% of soybean [*Glycine max* (L.) Merr], 74% of cotton, and 33% of corn (*Zea mays* L.) planted in the United States were glyphosate-tolerant varieties (*1, 51*).

Throughout the growing season, glyphosate-tolerant cotton is sprayed with multiple applications of glyphosate and is also treated with additional pesticides that may include the herbicide fluometuron. Fluometuron is used to control grass and broadleaf weeds in cotton. The potential for injury to subsequent crops (*52*) or groundwater contamination (*17*) caused by excessive residues or weed control failure due to excessive degradation makes knowledge of fluometuron soil persistence necessary.

Fluometuron in soil is dissipated by co-metabolic microbial degradation, creating the

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primary metabolites desmethyl-fluometuron and trifluoromethylphenylurea (*36*). The fungus *Rhizoctonia solani* is capable of degrading fluometuron; however, complete degradation of the herbicide was not observed in pure culture (*24*). When soil was amended with glucose and yeast extract, the degradation of fluometuron was enhanced (*36*).

It is unknown if the addition of other pesticides, such as glyphosate affect the degradation of fluometuron either in soil or in pure culture. Previously published research suggests that the response of soil microbial communities to pesticides is altered by the presence of glyphosate in the soil (*10, 13, 53*). In addition, simultaneous application of glyphosate and atrazine resulted in enhanced microbial activity (*15*) and varied atrazine degradation (*14*).

The objectives of this research were to describe 1) the influence of glyphosate and fluometuron on microbial activity and fluometuron degradation in bulk soil and 2) the effect of glyphosate on fluometuron degradation and growth of *R. solani* in liquid culture.

MATERIALS AND METHODS

Soil. The soil used in this study was a Weswood silty clay loam (fine-silty, mixed superactive, thermic Udifluventic Haplustept) with pH 8.0 with 32% clay and 1.4 % organic matter. Bulk soil was collected from a fallow field that was previously planted to cotton, air-dried and passed through a 2-mm sieve prior to the beginning of the experiment.

Herbicides. Commercial formulations of glyphosate (Roundup WeatherMAX, Monsanto Company, St. Louis, MO) and fluometuron (Cotoran 4L, Griffin L.L.C., Valdosta, GA), were used. Pesticides will be referred to by their common names throughout this paper. Herbicide application rates are listed in the table on page 27. Herbicide rates were consistent with recommended application rates and adjusted by an effective interaction depth of 2 mm for glyphosate (*13*) or 50 mm for fluometuron (*54*). The effective soil interaction depth represents the depth in the soil profile to which the herbicide is expected to be found following a field application. Adjusting the herbicide rate by this depth results in a more realistic concentration of the herbicide in soil.

Soil microbial respiration. Thirty grams of dry weight equivalent soil were placed into 50-mL plastic beakers with holes for drainage and water absorption that were covered with filter paper. Fifteen mL deionized H_2O were added to gas-tight chambers and samples were placed in the chambers. Soil was incubated at 23 C $\pm$ 0.62 to allow stabilization of the soil microbial biomass following the initial flush of activity after re-wetting (*28*). After 10 days, soils were fortified with 1.5 mL of herbicide solution (**Table 4**) and chambers were flushed with N_2 to remove CO_2. Carbon dioxide concentration was determined every h for 33 d using an infrared gas analyzer (ADC 225MK3, BioScientific Ltd., Great Amwell, England).

Fluometuron degradation in soil. Ten grams of dry weight equivalent soil were placed into 50-mL plastic beakers and 2.5 mL deionized H_2O were added (60% water-filled pore space). Soil was incubated at 25C $\pm$ 2 with a container of 10 mL 1 M KOH for 5 d in 1-L gas-tight containers to allow stabilization of the soil microbial biomass

(*28*). Potassium hydroxide traps were then removed and soil samples were fortified with 0.5 mL herbicide solution (**Table 4**). Samples were returned to gas-tight containers with 15 mL fresh KOH solution, which were replaced at 10 d intervals during the experiment to prevent excessive accumulation of CO_2 in the containers. Fluometuron was extracted from soil samples after 1, 3, 6, 10, 15, 20, 30, or 40 d of incubation at 25 C $\pm$ 2.

Fluometuron degradation by *R. solani*. *Rhizoctonia solani* (anastomosis group AG4) collected in Brazos County, TX was grown in a liquid medium containing 20 g glucose, 1 g NH_4NO_3, 0.9 g K_2HPO_4, 0.2 g KCl, 0.2 g $MgSO_4$-$7H_2O$, 0.002 g $FeSO_4$-$7H_2O$, 0.002 $ZnSO_4$-H_2O, and 0.002 g $MnCl_2$ per L (*55*). One hundred mL of this medium were supplemented with fluometuron alone or in combination with glyphosate (**Table 4**). Herbicide concentrations represent the concentration of herbicide in the amount of Weswood silty clay loam needed to hold 100 mL solution at 33% gravimetric water content. Control treatments of 1) non-inoculated media, 2) non-supplemented media and 3) non-inoculated, non-supplemented media were included.

Cultures were maintained at 37 C on an orbital shaker (G24, New Brunswick Scientific, Edison, NJ) for 1, 3, 6, 10, 15, or 20 d. Following incubation, cultures were dried, accumulated biomass was quantified, and the fluometuron concentration in biomass was quantified. Prior to desiccation, a 1-mL aliquot of the liquid media was removed to determine the fluometuron content of the media.

Fluometuron extraction and analysis. Fluometuron was extracted from soil and fungal biomass samples using accelerated solvent extraction (ASE) methodology (*56*). For soil samples, 1 g Hydromatrix was thoroughly mixed with each 10 g soil sample

Table 4. Herbicide treatments evaluated in soil [a] respiration and degradation experiments.

Herbicide[b]	kg a.i. ha^{-1}	µg a.i. g $soil^{-1}$ [c]	µL herbicide g $soil^{-1}$ [d]	µg a.i. mL $media^{-1}$ [e]	µL herbicide mL $media^{-1}$
Fluometuron	2.25	3.5	0.007	11.7	0.023
fluometuron + 1X glyphosate	2.25 + 1.25	3.5 + 49.7	0.007 + 0.092	11.7 + 146	0.023 + 0.27
fluometuron + 2 X glyphosate	2.25 + 2.5	3.5 + 99.5	0.007 + 0.184	11.7 + 292	0.023 + 0.54

[a] Soil was Weswood clay loam collected from a fallow field previously planted to cotton.

[b] Pesticides were applied as formulated products: fluometuron, Cotoran 4L (480 g ai/L) and glyphosate, Roundup WeatherMax (540 g ae/L).

[c] Herbicide rates represent the soil concentration of herbicides assuming effective interaction depths of 50 mm for fluometuron and 2 mm for glyphosate.

[d] Rate of application of formulated product: Cotoran 4L, 4.67 L/ha and Roundup WeatherMax, 7.01 L/ha.

[e] Herbicide concentrations based on volume soil solution in 330 g soil at 80% .

volumetric water holding capacity.immediately prior to extraction, and samples were transferred to ASE cells assembled with filter at bottom. Samples were extracted by the ASE with methanol at 50 C during three 5-min. static cycles. Dessicated fungal biomass was extracted similarly, except that no Hydromatrix was added and only two static cycles were used. Concentrations of fluometuron were quantified using high performance liquid chromatography-photodiode array detection. The mobile phase was 50:50 acetonitrile:water, the injection volume was 10 µL, and the flow rate was 0.2 mL min^{-1}. Samples were analyzed at 243 nm.

Data analysis. Treatments were replicated four times in the respiration and soil degradation experiments and three times in the *R. solani* experiment. All data were analyzed using Statistical Analysis Systems v. 9.1 (SAS Institute, Inc., Cary, NC). Mixed models (*57*) were used to determine and separate treatment means and slope parameters by pair-wise comparisons ($\alpha = 0.05$). Carbon mineralization data were analyzed appropriately for repeated measurements.

RESULTS AND DISCUSSION

Soil microbial respiration. Fluometuron alone had little effect on hourly C-mineralization. Carbon mineralization was generally increased when fluometuron was added with glyphosate at the 1X rate, and an additional increase was observed when fluometuron was applied with the 2X rate of glyphosate (**Figure 6**). Maximum hourly mineralization for all treatments occurred approximately 210 h (9 d) after herbicide addition. However, there were considerable fluctuations in the rate of CO_2 production

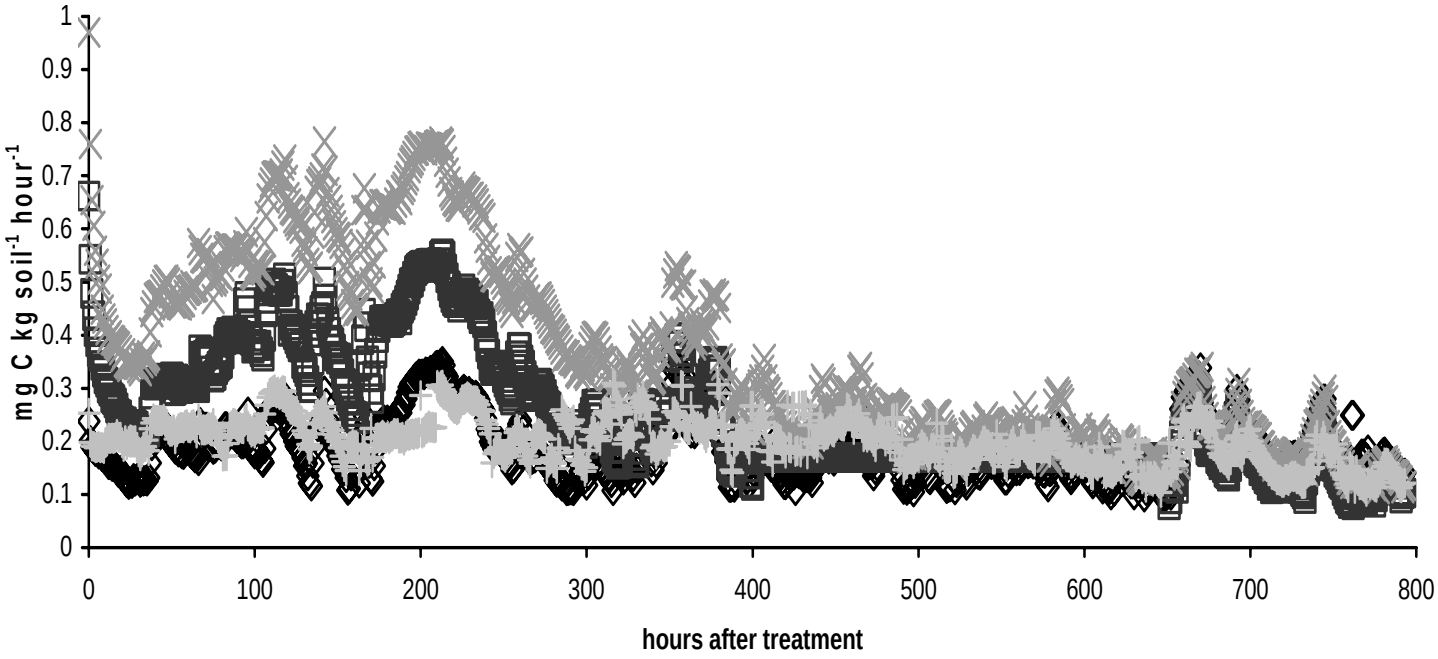

Figure 6. Carbon mineralized during 793 h (33d) following addition of 2.25 kg ai/ha fluometuron (◊), 2.25 kg fluometuron + 1.25 kg ae/ha glyphosate (□), 2.25 kg fluometuron + 2.5 kg ae/ha glyphosate (x), or no herbicide (+). Data are means of four replicates.

during the first 400 h (16 d) of the experiment. This fluctuation has been observed in other experiments (R. Haney, personal communication) and is likely due to inherent cycles of the microbial community. The frequent sampling in this experiment allowed these observations, which are in contrast to some previous reports of C-mineralization following glyphosate application, which indicate a single maxima in daily respiration 2 d after application (*10, 13*). By approximately 450 h (19 d), C-mineralization in all treatments had returned to approximately basal levels of respiration, indicating that the herbicides applied were no longer influencing soil microbial activity. Haney et al. (*13*) reported that C-mineralization following the application of glyphosate alone returned to background levels after 14 d.

Rates of C-mineralization were analyzed by comparing the linear and quadratic functions of the lines representing CO_2 accumulated during the experiment (**Table 5**). Trends observed in cumulative C-mineralization were similar to those in hourly mineralization. Soil treated with fluometuron and 2.5 kg/ha glyphosate produced the greatest amount of CO_2 over the course of the experiment (**Figure 7**). When fluometuron was applied with 1.25 kg/ha glyphosate, the amount of CO_2 produced was less than in soil treated with fluometuron and 2.5 kg/ha glyphosate, but greater than in soil treated with only fluometuron. The total amount of C-mineralized in soil treated with fluometuron was similar to the amount of C-mineralized in untreated soil.

The increase in total C-mineralized from each treatment was approximately equal to the relative amounts of glyphosate added in each treatment (**Table 6**). This indicates

Table 5. Slope parameters of modeled cumulative C-mineralization[a, b].

Treatment[b]	Intercept parameter[c]	Linear parameter	Quadratic parameter
fluometuron	-1.89 a	0.22 a	-0.0001 a
fluometuron + 1X glyphosate	-4.04 b	0.44 b	-0.0003 b
fluometuron + 2X glyphosate	-7.93 c	0.68 c	-0.0004 c
untreated	-1.15 a	0.24 a	-0.0001 a
P-value	0.008	<0.0001	<0.0001

[a]Values within a column followed by the same letter are not significantly different at ($P<0.05$) according to Tukey's multiple pair-wise comparisons.

[b]Parameters for polynomial equation: $y = \beta_0 + \beta_1 x + \beta_2 x^2$; β_0 = intercept, β_1 = linear, β_2 = quadratic.

[c]Herbicide rates are listed in Table 4.

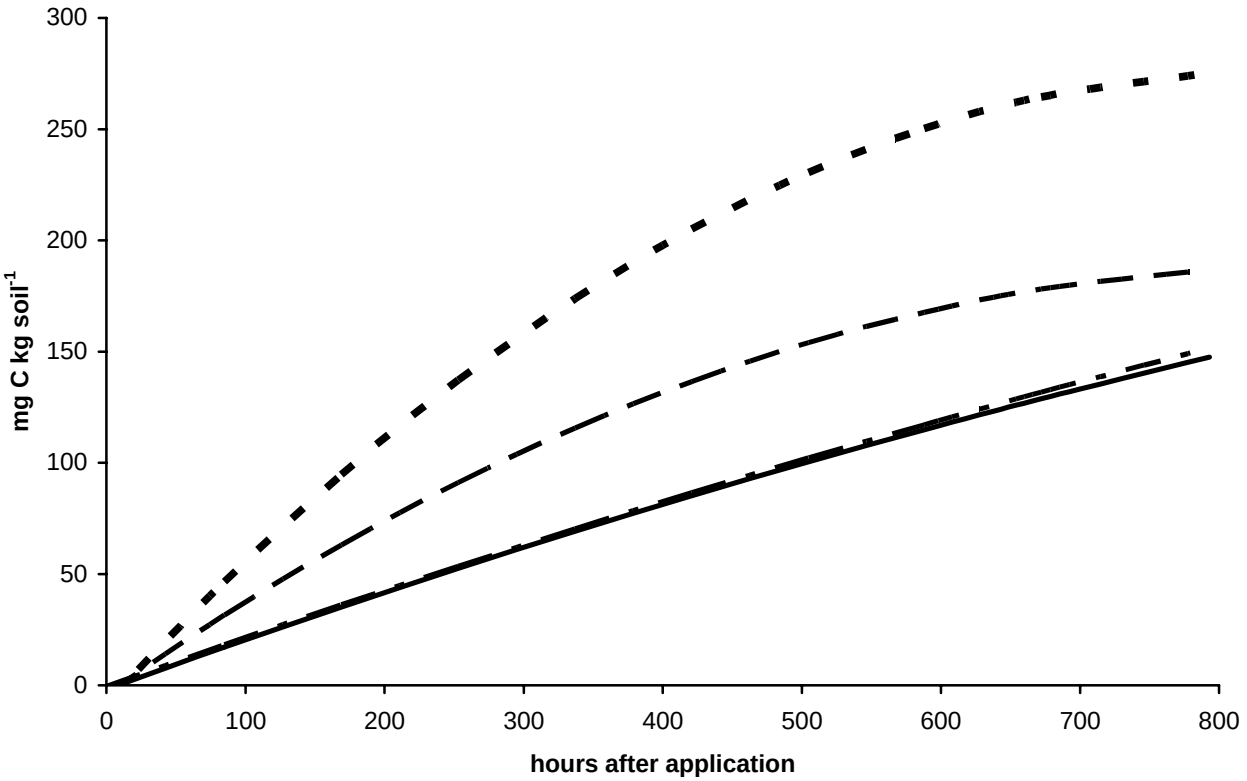

Figure 7. Fitted equations representing cumulative C mineralization 793 h (33 d) following addition of 2.25 kg ai/ha fluometuron (——), 2.25 kg fluometuron + 1.25 kg ae/ha glyphosate (— —), 2.25 kg fluometuron + 2.5 kg ae/ha glyphosate (- - -), or no herbicide (— - —). Equation parameters are listed in Table 2.

Table 6. Comparison of C-added as glyphosate in each treatment and C-mineralized from each treatment.

Treatment[a]	C-added as glyphosate mg C kg soil^{-1}	C-added as glyphosate ratio	C-mineralized mg C kg soil^{-1}	C-mineralized ratio
fluometuron	0	0	147.4	1
fluometuron + 1X glyphosate	10.6	1	193.2	1.3
fluometuron + 2X glyphosate	21.2	2	284.4	1.9
untreated	0	0	151.5	1

[a]Herbicide rates are listed in Table 4.

that the addition of glyphosate is directly related to increased C-mineralization due to either degradation of the herbicide (*13*) or a priming effect of glyphosate (*58*).

Fluometuron degradation in soil. No fluometuron residues were detected in untreated soil samples (data not shown). The concentration of fluometuron remaining in the soil was similar among all treatments one, three, six, and thirty days after application (**Table 7**). Ten, fifteen, twenty, and forty days after herbicide application, less fluometuron was present in soil treated with fluometuron plus the 2X rate of glyphosate relative to soil treated with fluometuron alone. Ten, twenty, and forty days after application, soils treated with fluometuron plus the 2X rate of glyphosate also contained less fluometuron than soils treated with fluometuron plus the 1X rate of glyphosate. Forty days after treatment, the concentration of fluometuron remaining was 32% of applied in soils treated with fluometuron alone, 29% in soils treated with fluometuron plus 1.25 kg/ha glyphosate, and 24% in soil treated with fluometuron plus 2.5 kg/ha glyphosate.

Soil amendments commonly affect pesticide degradation. For example, Bozarth and Funderburk (*36*) reported that fluometuron degradation was more rapid in soils amended with glucose than in non-amended soils. However, the influence of amendments such as glucose or glyphosate on pesticide degradation is also influenced by the nutrient availability of a soil (*58*).

First-order kinetics were used to describe fluometuron degradation in this experiment, as well as others (*17*, *59, 60, 61*). Linear regression of the natural log of concentration remaining/initial concentration and days after application are presented in **Figure 8**. The

Table 7. Fluometuron remaining in soil treated with fluometuron applied alone or with glyphosate after incubation at 27 C[a].

	Days of incubtaion							
Treatment[b]	1	3	6	10	15	20	30	40
				μg fluometuron g soil^{-1}				
fluometuron	3.2	3.1	3.0	2.8 a	2.5 a	2.3 a	1.7	1.1 a
fluometuron + 1X glyphosate	2.9	3.1	2.9	2.8 a	2.4 ab	2.2 a	1.4	1.1 a
fluometuron + 2X glyphosate	3.2	2.9	2.6	2.3 b	2.1 b	1.4 b	1.3	0.84 b
Pr >F	NS	NS	NS	0.0169	0.0206	0.0019	NS	<0.0001

[a]Values within a column followed by the same letter are not significantly different at ($P < 0.05$) according to Tukey's multiple pair-wise comparisons. NS indicates $Pr > F > 0.05$.

[b]Herbicide rates are listed in Table 4.

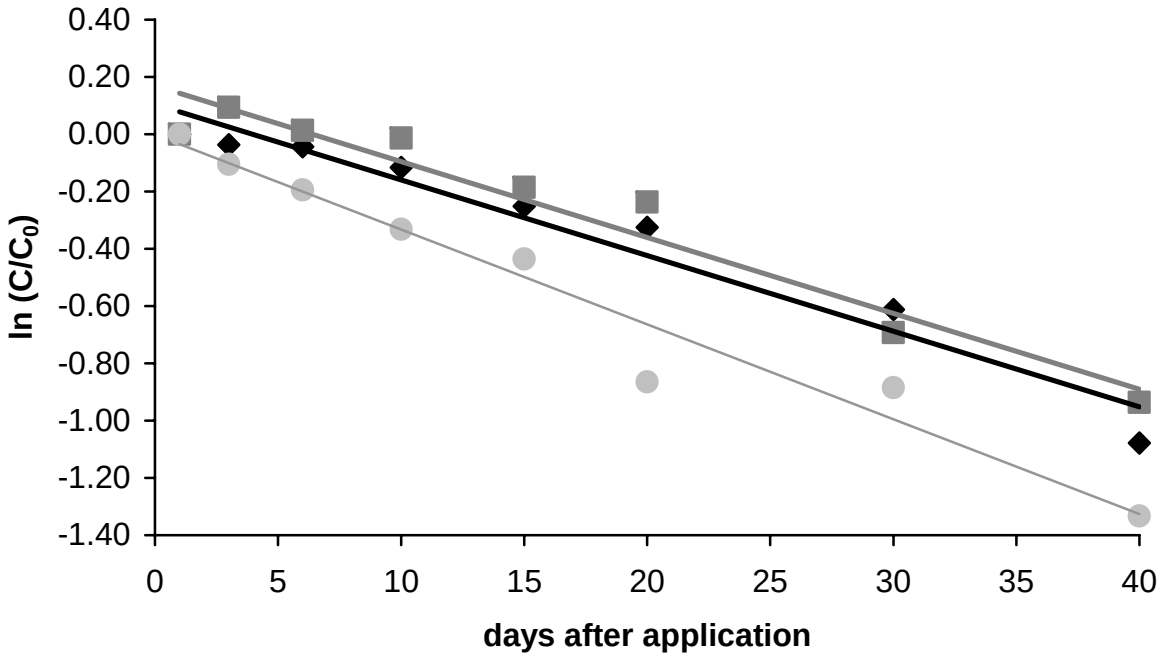

Figure 8. First-order rate plots for degradation of fluometuron applied alone (2.25 kg ai/ha; ♦) and with 1.25 kg ae/ha glyphosate (■) or 2.5 kg ae/ha glyphosate (●). Fitted equations are: y = -0.03x+0.13, fluometuron alone; y = -0.03x+0.17, fluometuron + 1X rate of glyphosate; y = -0.03x-0.001, 2X rate of glyphosate.

Table 8. First-order rate constant (*k*), half-life ($t_{1/2}$), and coefficient of determination (R^2) of fluometuron in soils treated with fluometuron alone or with glyphosate[a].

Treatment[b]	*k*	$t_{1/2}$	R^2
	(d^{-1})	(d)	
fluometuron	0.025 a	28.6 a	0.81
fluometuron + 1X glyphosate	0.026 a	26.9 ab	0.71
fluometuron + 2X glyphosate	0.033 b	21.2 b	0.92

[a]Values within a column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's multiple pair-wise comparisons.

[b]Herbicide rates are listed in Table 1.

calculated degradation rate constant, half-life, and coefficient of determination for each line are presented in **Table 8**. The half-life calculated for fluometuron alone was 28.6 d. This is greater than the half-life of 18 d reported by Mueller et al. (*17*) and substantially shorter than the half-life of 49 d reported by Brown et al. (*59*), but within the range of half-lives reported for fluometuron in 8 soils by Willian et al. (*60*). Differences in these reported half-lives may be due to differences in organic matter content, pH, microbial biomass, or microbial activity of the soil used in each experiment (*17*, *59, 61*).

When fluometuron was applied with a 1X rate of glyphosate (**Table 8**), the rate of fluometuron degradation was similar to fluometuron applied alone. However, fluometuron degradation was faster in soils that were treated with fluometuron plus the 2X rate of glyphosate relative to fluometuron applied alone. Soils treated with the 2X rate of glyphosate also exhibited greater soil microbial activity.

Glyphosate application has been associated with increased microbial activity (*10, 13*) as well as numerically greater microbial populations (*62*). Fluometuron degradation has been positively correlated with microbial respiration, as well as soil microbial biomass in other studies (*17*, *61*). Therefore, it is possible that the enhanced fluometuron degradation observed when glyphosate was applied with fluometuron is related to a glyphosate-induced increase in the population of microorganisms able to degrade fluometuron.

Fluometuron degradation by *R. solani.* There were no differences among herbicide treatments in the fluometuron concentration of *R. solani* biomass at any extraction time (data not shown). However, after 10 and 20 d, the concentration of fluometuron detected

in the medium was greater when glyphosate was included at either the 1X or 2X rate than when fluometuron was applied alone (**Table 9**). After 20 d, no fluometuron was detected in the medium that did not contain glyphosate. It is important to note that lack of detection does not equal complete degradation of fluometuron, rather, the concentration present was lower than the limit of quantitation (0.39 μg/mL). When glyphosate was included in the media, the amount of fluometuron remaining was 63% and 85% of the amount detected the first day after the herbicides were added in the low glyphosate and high glyphosate treatments, respectively.

Treatments that included glyphosate also suppressed the growth of *R. solani* biomass. (**Figure 9**). After 20 days, medium containing fluometuron alone supported fungal growth similar to media containing no herbicides. Fungal growth in media containing fluometuron and either rate of glyphosate was not statistically greater than the non-inoculated samples.

The reduction in fungal biomass may be related to the presence of proprietary adjuvants in the glyphosate formulation. Lee et al. (*63*) reported reduced growth of *Sclerotinia sclerotiorum* mycelia in the presence of a Roundup formulation blank. In addition, this finding is supported by the fact that *R. solani* grows poorly in the presence of the amino acids alanine and leucine (*64*). Glyphosate is an amino acid analog with a chemical structure very similar to the amino acid glycine.

Even though the simultaneous addition of glyphosate enhanced degradation of fluometuron in soil, degradation by *R. solani*, an organism known to metabolize

Table 9. Percent fluometuron remaining in minimal medium supplemented with fluometuron alone or with glyphosate following incubation at 27 C[a].

	Days of incubation					
Treatment[b]	1	3	6	10	15	20
fluometuron	115.0	82.2	90.8	76.7 a	65.0	0.0 a
fluometuron + 1X glyphosate	100.5	95.7	101.8	99.3 b	99.2	94.5 b
fluometuron + 2X glyphosate	109.2	100.0	99.6	97.3 b	96.0	92.1 b
Non-inoculated control	101.8	100.0	97.6	102.8 b	99.8	91.1 b
Pr >F	NS	NS	NS	0.0159	NS	0.0009

[a]Values within a column followed by the same letter are not significantly different at ($P<0.05$) according to Tukey's multiple pair-wise comparisons.

[b]Herbicide rates are listed in Table 4.

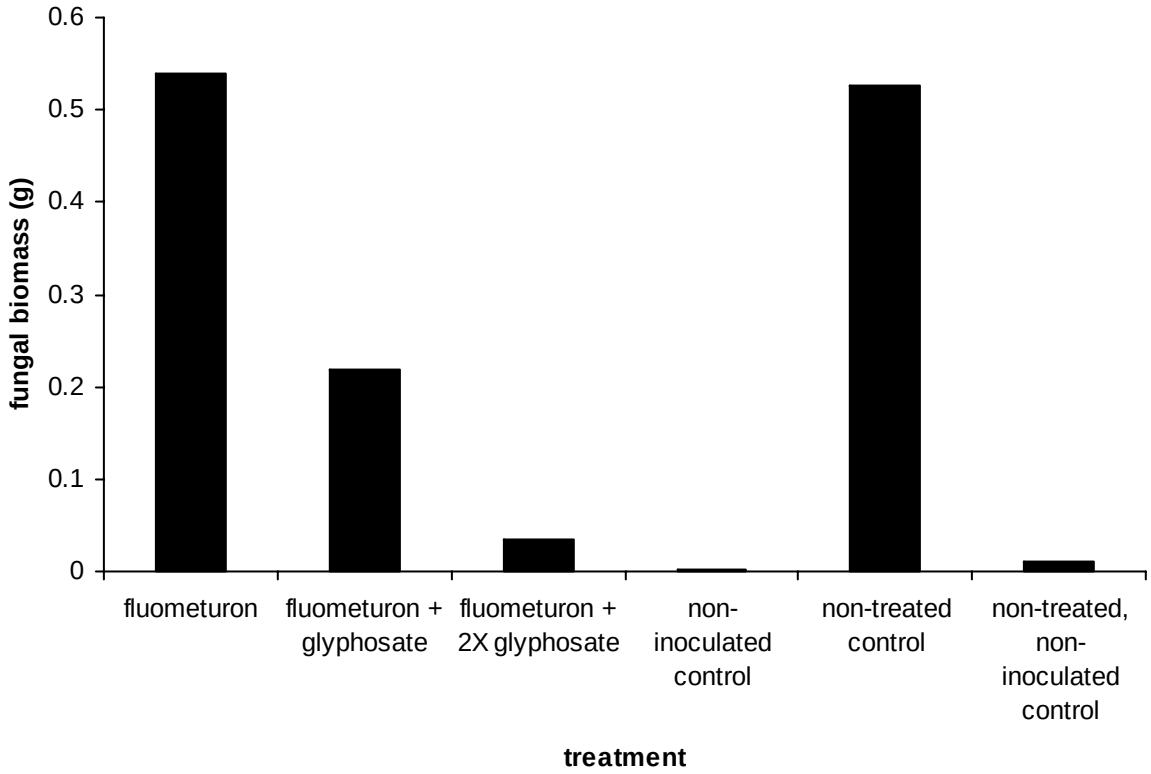

Figure 9. *Rhizoctonia solani* biomass accumulated following 20 d of growth in minimal media containing 20 g glucose, 1 g NH_4NO_3, 0.9 g K_2HPO_4, 0.2 g KCl, 0.2 g $MgSO_4$-$7H_2O$, 0.002 g $FeSO_4$-$7H_2O$, 0.002 $ZnSO_4$-H_2O, and 0.002 g $MnCl_2$ per L. Bars labeled with similar letters are similar according to pair-wise t-tests ($\alpha = 0.05$).

fluometuron was reduced when grown in media containing glyphosate. This is supportive of other research which indicates that glyphosate application does not increase the occurrence of soybean diseases caused by *R. solani* (*41*). However, increased growth of other pathogens in response to glyphosate has been reported (*23, 65*).

The different responses noted for microbial degradation of fluometuron when applied with glyphosate to soil and in pure culture demonstrate the complex nature of microbial interactions with regard to pesticide degradation. This complexity is enhanced when pesticide degradation is considered in multi-pesticide systems, which are more representative of agricultural practices. Additional research is needed to further elucidate the effects of glyphosate-based weed management programs on soil microorganisms.

CHAPTER IV

SOIL MICROBIAL RESPONSE TO REPEATED GLYPHOSATE APPLICATIONS

INTRODUCTION

The use of glyphosate-tolerant crops has been widely adopted in the United States. Currently, 96% of soybean, 74% of cotton, and 33% of corn planted in the United States are glyphosate-tolerant varieties (*1, 51*). The average glyphosate-tolerant cotton crop is treated with glyphosate 2.1 times each year (*1*). However, glyphosate may be applied up to four times during the cotton growing season (*8*). In addition, glyphosate may also be applied prior to planting or used as a harvest aid.

Glyphosate is the most widely used pesticide in the world (*66*), and there have been numerous studies conducted to evaluate the environmental impacts of single glyphosate applications (*10, 12, 13, 67*). However, there is considerably less information available regarding the environmental impacts of repeated glyphosate use in annual crops. Repeated applications of a pesticide may have a greater impact on soil microorganisms than a single application (*19, 68*). Additionally, scientists in Brazil reported reduced C-mineralization and extended herbicide half-life following two, three, or four applications of glyphosate relative to one application (*21*).

The experiments presented here were conducted with the goals of studying the effect of 1, 2, 3, 4, or 5 applications of glyphosate on: 1) soil microbial community composition and 2) the degradation and distribution of ^{14}C-glyphosate in soil.

MATERIALS AND METHODS

Glyphosate dissipation and biomass incorporation. Weswood silt loam (fine-silty, mixed superactive, thermic Udifluventic Haplustept) with 1.7% organic matter and pH 7.9 was collected from an area with no record of glyphosate application during the previous two years. Soil was air dried and passed through a 2-mm sieve. Four mL deionized H_2O (20% volumetric water content) were added to 20 g dry weight equivalent soil in 50-mL plastic beakers and the soil was incubated at 27 $\pm$ 2 C with 10 mL 1 M NaOH in 1-L gas-tight containers to allow stabilization of the soil microbial biomass (*28*). After 5 d, NaOH traps were removed and soils were fortified with 0.4 mL glyphosate solution.

Samples were treated with glyphosate (RoundUp WeatherMAX, Monsanto Company, St. Louis, MO) 1, 2, 3, 4, or 5 times. At the time of the first application, all treatments received 49 μg ae g soil^{-1}. This represents the concentration of glyphosate present following a field application in a 2-mm soil interaction depth (*10*). Soils treated only once received 277.5 Bq g soil^{-1} of ^{14}C-glyphosate (specific activity 81.4 MBq/mM, labeled at the phosphonomethyl carbon). Samples were returned to gas-tight containers with 20 mL fresh NaOH solution. Additional glyphosate applications of 49 μg glyphosate g soil^{-1} were made at 2-week intervals, with the final glyphosate application for each treatment being radiolabeled glyphosate.

Glyphosate mineralization was determined by sampling NaOH 1, 2, 3, 7, and 14 d after the final herbicide addition. A 1-mL aliquot of NaOH was added to 5 mL EcoLite(+) (MP Biomedicals, Irvine, CA) and $^{14}CO_2$ evolution was quantified using liquid

scintillation spectroscopy (LSS). Distribution of the remaining glyphosate was determined 3, 7, and 14 days after application (DAA). ^{14}C-residues were extracted from soil using a wrist-action shaker (Burrell Scientific, Pittsburgh, PA) rotating approximately 300 revolutions per minute with 20 mL of 0.1 M NaOH (*69*). After 24 h, samples were centrifuged at 13,000 X g for 15 min. This speed was selected to approximate the glyphosate that would be present in soil solution at the permanent wilting point (-1,500 kPa) (*70*). A 1-mL aliquot of the supernatant was added to 5 mL EcoLite(+) and ^{14}C-residue present in the solution were quantified using LSS. The soil was dried, ground, and a 0.5 g sub-sample was weighed onto Whatman 1 qualitative filter paper (Whatman International, Ltd, Maidstone, England). The sub-samples were combusted in an R. J. Harvey Biological Oxidizer (R. J. Harvey Instrument Corporation, Tappan, NY) for 4 min. $^{14}CO_2$-residues were captured in Harvey Cocktail (R. J. Harvey Instrument Corporation, Tappan, NY) and LSS was used to determine the amount of ^{14}C remaining in the soil.

Glyphosate incorporation into soil microbial biomass (SMB) was determined using the chloroform fumigation-incubation method (*33*). $^{14}CO_2$ evolved during 10 d of incubation following chloroform fumigation was determined from a 1-mL aliquot of NaOH in EcoLite (+) using LSS. ^{14}C-glyphosate content of SMB was calculated by dividing the amount of ^{14}C recovered following fumigation by a *k* value of 0.41 (*71*) without subtracting a control (*72*).

Each treatment was repeated four times. $^{14}CO_2$ evolution was fitted to the first-order kinetics equation using non-linear regression (Statistical Analysis Systems version 9.1):

$$Y = a(1-e^{-kt})$$

where *a* is the maximum ^{14}C-mineralized (% of applied), *k* is the first-order rate constant (day^{-1}), *t* is time (days), and Y is 14C-mineralized at time *t*. Mixed models (Statistical Analysis Systems version 9.1) were used to determine treatment means for *k* values and ^{14}C recovery from soil SMB. Means were separated according to Tukey's pair-wise comparisons ($\alpha = 0.05$).

Soil microbial community composition. Ten g dry weight equivalent soil previously described was placed in 50-mL plastic beakers. Glyphosate was applied in a 3 mL solution (33% volumetric water content) at a rate of 49 μg ae g $soil^{-1}$. Soil was placed in 1-L air-tight chambers with 1M KOH to trap evolved CO_2 and incubated at 27 $\pm$ 2 C. Two, four, six, and eight weeks after the initial glyphosate applications, an additional 49 μg g $soil^{-1}$glyphosate were added in 0.5 mLs of solution, to create soil samples that received 1, 2, 3, 4, or 5 applications of glyphosate.

Fatty acid methyl esters (FAMEs) were used to evaluate soil microbial community composition three, seven, and fourteen d after the final glyphosate application (DAA). Soil samples were collected and stored at - 4 C until the FAME analysis was conducted (30 to 90 d following sample collection). The method outlined by Schutter and Dick (*73*) was used to extract FAMEs from each soil sample. Briefly, 3 g of soil were extracted with 0.2 M KOH at 37 C for 1 h and 3 mL 1.0 M acetic acid was added to neutralize the pH. Ten mL hexanes were added to the sample, which was then centrifuged at 1000 X g for 20 min. An aliquot of the hexane layer was removed and evaporated under N_2.

Extracted samples were sent to the University of Delaware Plant and Soil Sciences Department for analysis using an Agilent model 6890 gas chromatograph with flame ionization detector (Agilent, Wilmington, DE). Two mL of each sample were injected into a Hewlett Packard (Agilent) Ultra 2 column 25 m x 0.20 mm x 0.33 μm with a 100:1 split ratio and flow rate of 0.6 ml/min using hydrogen as the carrier gas. The injection temperature was 250 C, and the detection temperature was 300 C. The initial oven temperature was 170 C (hold for 0 min) and ramped at 5 C/min to a final temperature of 300 C, for a total run time of 12 min. Peaks were named using the Sherlock Eukary program (MIDI, Inc., Newark, DE).

Each treatment was replicated four times. Prior to data analysis, FAMEs present in fewer than 5% of samples (*74*) and greater than 20 carbons in length were deleted from the analysis (*75*). Any values beyond 3 standard deviations were replaced with the appropriate mean and data were relativized to improve CV and skewness of data (*74*). Permutation-based multivariate analysis of variance (PerMANOVA) in PC-ORD v.5 (MjM Software Design, Gleneden Beach, OR) was used to evaluate the significance of the effect of glyphosate application on FAME composition at each sampling time based on Sørensen's distance (*74*). Non-metric multidimensional scaling (NMS) was performed using PC-ORD v.5 on data sets containing FAMEs extracted from samples 3, 7, or 14 d after the final glyphosate applications based on Sørensen's distance and the "slow and thorough" autopilot mode of PC-ORD. Canonical correspondence analysis (CCA) of FAMEs and ^{14}C-data was performed using PC-ORD v.5 with LC scores plotted and axes scaled according to Hill's scaling (*74*). Analysis of variance was

performed on individual FAMEs using mixed models (Statistical Analysis Systems version 9.1, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Glyphosate dissipation. Average recovery of mineralized, bound, and extractable ^{14}C-residue was 85.4%. ^{14}C mineralized from soil treated with a single glyphosate application was similar to ^{14}C-mineralization previously reported (*21, 67*). Glyphosate mineralization followed first-order kinetics, regardless of the number of glyphosate applications (**Figure 10**). The first-order rate constant was greater for 1 application than for five applications (**Table 10**), indicating that slower mineralization of glyphosate occurred when more glyphosate was applied. The total amount of ^{14}C mineralized 14 DAA was reduced when glyphosate was applied 4 or 5 times relative to the cumulative amount of ^{14}C mineralized following 1, 2, or 3 applications (data not shown). Likewise, de Andréa et al. (*21*) reported a decrease in $^{14}CO_2$ production following 2, 3, or 4 applications of glyphosate.

The ^{14}C-glyphosate used in this experiment was labeled at the phosphonomethyl-C. This carbon molecule is also present in the primary metabolite of glyphosate, aminomethylphosphonic acid (AMPA) and is only released as CO_2 when AMPA is completely degraded to CO_2, NH_4^+, and inorganic P (*9*.). Therefore, the only source of $^{14}CO_2$ evolved was the complete degradation of the herbicide by this pathway.

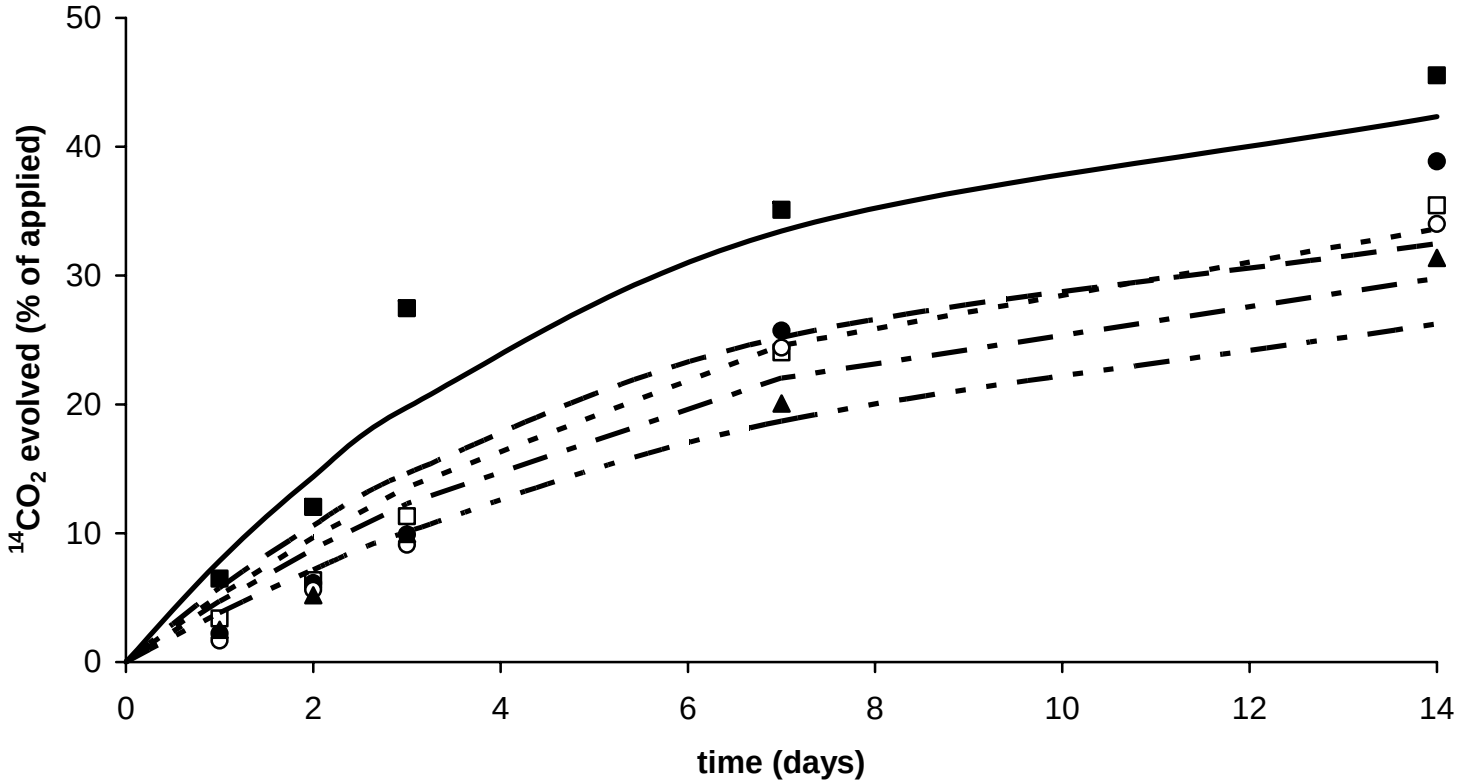

Figure 10. Mineralization of glyphosate in soil: 1 application ——■——; 2 applications — —□— —; 3 applications - - -●- - -; 4 applications — - ○ - —; 5 applications — - -▲- - -. Lines fitted to first-order kinetics model: $Y = a(1-e^{-kt})$.

Table 10. Rate constant (*k*) at coefficient of determination ($R^{2)}$ for glyphosate mineralization in soil.[a]

No. glyphosate applications	*k*	R^2
1	0.21 a	0.85
2	0.18 ab	0.79
3	0.14 ab	0.94
4	0.16 ab	0.91
5	0.13 b	0.91
Pr>F	0.0364	--

[a]Means within a column followed by similar letters are not significantly different according to Tukey's test ($P \leq 0.05$).

However, it is possible that some degradation of glyphosate occurred resulting in the release of CO_2 and leaving the metabolites AMPA or sarcosine (*9*, *76*). Some experiments evaluating the effect of glyphosate on C-mineralization have reported increased total C-mineralization following the application of glyphosate (*10, 13, 35, 53*). It is possible that the inability of these experiments to differentiate between complete degradation and incomplete glyphosate metabolism or mineralization of soil organic matter is one reason for seemingly contrasting results.

The percent of added ^{14}C-glyphosate bound to soil 3, 7, or 14 DAA was not affected by the number of glyphosate applications (**Table 11**). Similarly, de Andrea et al. (*21*) found that ^{14}C recovery from soils treated with 1, 2, 3, or 4 applications of ^{14}C glyphosate was similar 4 and 8 weeks following the final glyphosate application.

More applications of glyphosate resulted in more ^{14}C-residues available for extraction. Three and seven DAA, more ^{14}C-residues were extracted from soils with 4 or 5 applications relative to soils with only 2 applications. Fourteen DAA, more ^{14}C-residues were extracted from soils treated 4 or 5 times relative to soils treated once or twice.

More ^{14}C-residues were extracted from soil 3 DAA relative to 14 DAA. Similarly, more ^{14}C-residues remained bound to soil 3 DAA relative to 14 DAA. These results are likely due to more degradation of ^{14}C-glyphosate by the end of the experiment, removing the herbicide from the soil. Additionally, these data support the results of Schnurer et al. (*76*) who found that glyphosate is subject to microbial degradation when it is bound to soil.

Table 11. ^{14}C residues evolved as $^{14}CO_2$, bound to soil, or extracted from soil 3, 7, and 14 days after application (DAA) of ^{14}C-glyphosate[a].

	3 DAA			7 DAA			14 DAA		
	Evolved	Bound	Extracted	Evolved	Bound	Extracted	Evolved	Bound	Extracted
Applications	%								
1	27.5 aA	40.6 aA	35.2 abA	35.1 aAB	34.8 aA	26.7 abB	45.5 aB	24.1 aB	15.1 aC
2	11.3 bA	39.6 aA	28.1 aA	24.0 bB	35.2 aA	19.7 AB	35.4 abC	26.1 aB	10.6 aB
3	9.9 bA	35.4 aA	32.7 abA	25.7 bB	33.4 aA	25.2 abB	38.9 abC	24.5 aA	16.7 abC
4	9.1 bA	39.4 aA	39.7 bA	24.4 bB	35.5 aAB	32.3 bB	34.0 abC	27.4 aB	26.2 cC
5	9.9 bA	43.5 aA	41.4 bA	20.1 bB	34.2 aB	34.2 bB	31.4 bC	23.4 aC	23.5 bcC

[a]Means within a column followed by similar lowercase letter are not significantly different according to Tukey's test ($P \leq 0.05$). Means within a row followed by similar uppercase letters are not significantly different for each fraction according to Tukey's test ($P \leq 0.05$).

In general, when glyphosate was applied once or twice, more of the non-mineralized ^{14}C-residues were bound to the soil than extracted, whereas when glyphosate was applied 4 or 5 times, similar amounts of ^{14}C-residues were bound and extracted. The relative reduction of ^{14}C- residues bound to soil over time indicates complete degradation of the herbicide. However, the exchange sites where the herbicide had been adsorbed were possibly occupied by phosphate remaining from the degradation of previously applied glyphosate. The rapid binding characteristic of glyphosate is facilitated by the phosphonic acid moiety of the molecule (*9*), which would be the final portion of the herbicide molecule remaining on the adsorption site. Any subsequently added ^{14}C-glyphosate would not be likely to replace the remaining phosphate (*77*) and would therefore be available for extraction, which may have led to the increase in extractable ^{14}C-residues.

Soil microbial biomass incorporation of glyphosate. Incorporation of ^{14}C-residues into SMB was greater following 5 glyphosate applications than following the first application 3 and 7 DAA (**Figure 11**). This indicates that the microorganisms were better able to utilize glyphosate for growth-related metabolism following previous exposure to the herbicide. However, by 14 DAA there were no differences in the amount of ^{14}C-residues incorporated in the biomass. This suggested that while some of the ^{14}C was rapidly cycled through the organisms, some of the applied glyphosate could potentially be incorporated into more long-lived fractions of the soil organic matter. Charnay et al. (*78*) concluded that incorporation of herbicides into microbial biomass is an important step in the incorporation of herbicides into recalcitrant organic matter.

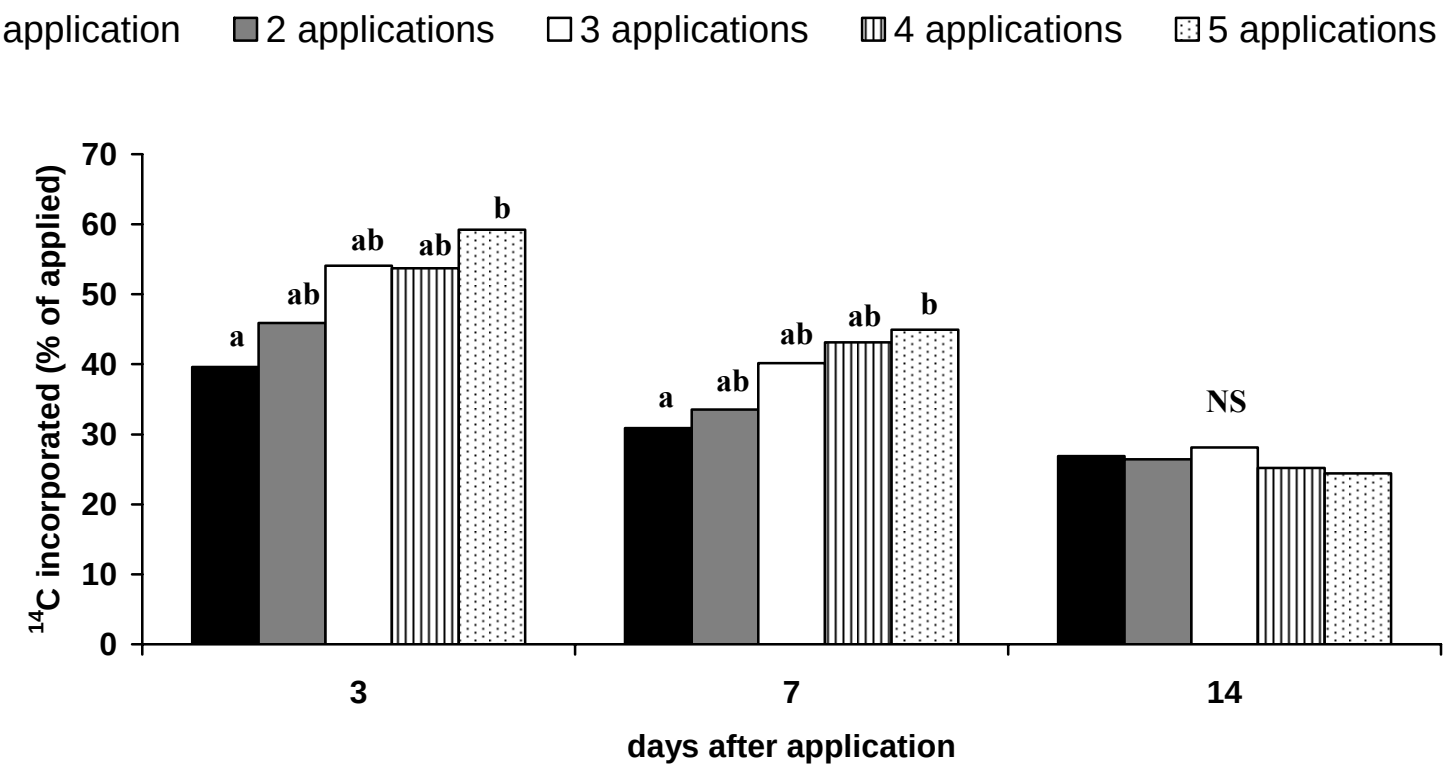

Figure 11. Incorporation of ^{14}C-glyphosate into soil microbial biomass after one to five applications 3, 7, and 14 days after application (DAA). Similar letters indicate similar means according to Tukey's test ($P < 0.05$).

From the biomass experiments, it appeared that glyphosate degradation may be related to growth-linked metabolism. Robertson and Alexander (*79*) suggested that the ability to utilize a pesticide as a C-substrate for metabolism indicates the potential for accelerated biodegradation. However, this was not the case in these experiments, which is in agreement with previous reports that glyphosate is degraded via co-metabolism (*9, 12*).

Other studies comparing repeated applications of glyphosate (*21, 79*) made multiple applications of the radio-labeled compound to the same soil. In these studies, radioactivity was only included in the final herbicide application. This made it possible to determine the difference in response to the herbicide at the time of the final application rather than the additive effect of all the applications.

Soil microbial community composition. The PerMANOVA results indicated a significant effect of treatment at all sample times on FAME profiles (3 DAA, $p \leq 0.047$; 7 DAA, $p \leq 0.032$; 14 DAA, $p \leq 0.008$). However, clear patterns of separation were not evident in the NMS plots at any DAA when all treatments were included in the analysis (Appendix). This is similar to previous research evaluating the effects of glyphosate on soil FAMEs, which reported little effect of glyphosate rates on FAME profiles (*80, 67*). However, when soil receiving one glyphosate application was compared to soil receiving five applications, separations between the two treatments were noted in the NMS plot (**Figure 12**). Likewise, CCA revealed a distinct separation of soils receiving one and five applications of glyphosate (**Figure 13**) but no separation was observed when other treatments were compared (Appendix).

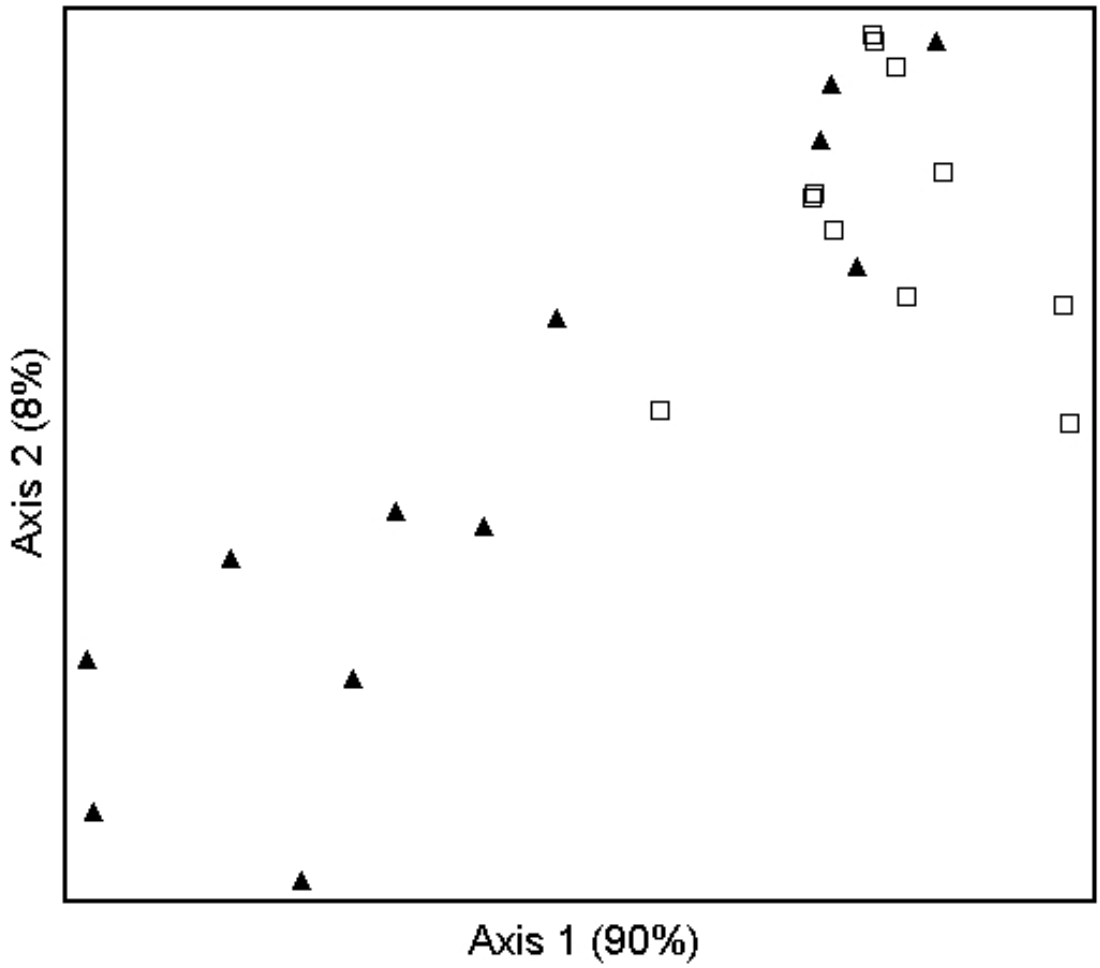

Figure 12. Nonmetric multidimensional scaling analysis of fatty acid methyl esters extracted from soil 3, 7, and 14 days after 1 (□) and 5 (▲) glyphosate applications. Numbers in parentheses represent the percent variance represented by each axis.

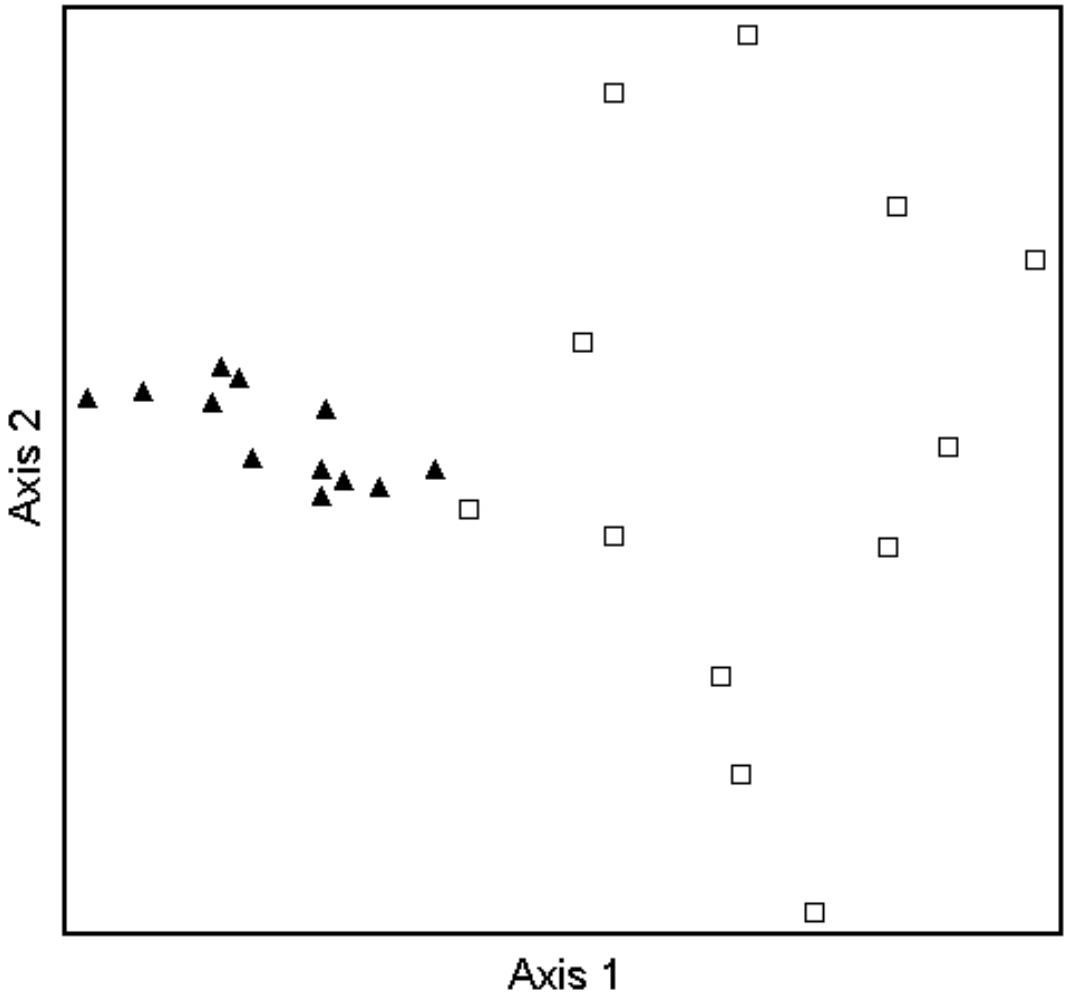

Figure 13. Canonical correspondence analysis of fatty acid methyl-esters extracted from soil 3, 7, and 14 days after 1 (□) and 5 (▲) glyphosate applications.

The total number of FAMEs present in each soil was similar 3, 7, and 14 DAA (data not shown). However, FAMEs from soils treated with glyphosate five times had less even species distribution according to Pielou's evenness index (PC ORD v5) than soils treated with glyphosate only once (data not shown). Comparison of the occurrence of individual FAMEs revealed no differences 3 DAA; however, differences were detected in FAMEs 7 and 14 DAA. FAMEs common to gram-negative bacteria were present in higher concentrations following 5 applications relative to 1, 2, 3, or 4 applications both 7 and 14 DAA (Appendix). These results are in contrast to Weaver et al. (*67*) who found differences 3 DAA, but not 7 or 14 DAA. Weaver et al. (*67*) also reported an increase in 20:0 FAMEs in response to glyphosate. This apparent increase in gram-negative bacteria is significant, as many microorganisms able to degrade glyphosate are gram-negative species (*11, 81, 82, 83*); however, some gram-positive bacteria are also able to degrade glyphosate (*83*). There were no increases in biomarkers for gram-positive bacteria or fungi in this experiment.

Other studies (*21, 67, 83*) have shown no correlation between soil microbial activity and other descriptive measurements of soil microbial function following 1, 2, 3, or 4 glyphosate applications. Similarly, no difference in soil microbial activity or function was detected in these experiments following the first four glyphosate applications. However, following the fifth glyphosate application, changes in ^{14}C-glyphosate mineralized, extracted, and accumulated in biomass were different relative to the first application. Shifts in FAME profiles followed a similar pattern, with separation among treatments occurring only when soils receiving one application were compared to soils

receiving five applications. These studies indicate that changes in the dissipation and distribution of glyphosate following intensive glyphosate use may be related to changes in the soil microbial community composition that occur in response to repeated applications of glyphosate.

CHAPTER V

ACCELERATED SOLVENT EXTRACTION OF FLUOMETURON FROM SELECTED SOILS*

INTRODUCTION

Analyte extraction from the soil matrix has commonly been accomplished using methods such as the solvent/shake flask method or Soxhlet extraction. These methods are effective; however, they consume substantial amounts of both time and solvents. Recently, accelerated solvent extraction (ASE) techniques have been developed for the extraction of various organic soil contaminants (*86, 87*).

Accelerated solvent extraction utilizes conventional organic solvents at high temperature and pressure to extract the analyte from the soil matrix (*86*). Increased temperature and pressure facilitate more rapid extractions using reduced volumes of solvent (*87*). Both of these characteristics make ASE a desirable application for analysis of environmental samples. However, there are few published methods for extracting pesticides from soil using ASE (*87, 88, 89, 90, 91*), and the authors know of no published methods for fluometuron.

Fluometuron is used in cotton production at rates of 1-2 kg/ha to control annual and perennial broadleaf weeds. Knowledge of the persistence of fluometuron in soil is

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beneficial because excessive residues may injure subsequent crops (*52*) and the herbicide has been cited as a potential groundwater contaminant (*17*). The influence of extraction temperature, solvent and repeated extraction cycles on ASE efficiency for removing the herbicide fluometuron from a silt loam soil treated with Cotoran 4L were evaluated.

MATERIALS AND METHODS

Soil preparation. The soil used in this study was a Weswood clay loam (fine-silty, mixed superactive, thermic Udifluventic Haplustept) collected from a fallow field that was previously planted to cotton and from a bermudagrass pasture. Pasture soils had pH 7.6 with 34% clay content and 6.2% organic matter. Field soils had pH 8.0 with 32% clay and 1.4 % organic matter. Soil was air-dried and passed through a 2-mm sieve prior to the beginning of the experiment. Ten grams of soil were mixed with 2 g washed silica sand (EMD Chemicals, Gibbstown, NJ) and placed in each extraction cell. Cotoran (Cotoran 4L® Griffin L.L.C., Valdosta, GA) was added to the top of each extraction cell in 2 mL methanol to achieve a fluometuron concentration of 4.1 μg/g soil. This concentration of fluometuron represents the amount of fluometuron present in the top 2 to 5 cm of soil following an application of Cotoran made at a recommended rate (*53*). Water was included in the mixture at 0.2% (v/v) to improve solubility of the formulated herbicide. Non-fortified samples were included in all experiments. Following application, fluometuron was allowed to equilibrate in the open cell for 24 h prior to extraction.

Extraction conditions. Optimum extraction conditions for ASE (Dionex Corp., Sunnyvale, CA) were determined in two separate experiments. The first experiment evaluated combinations of solvent (methanol or acetonitrile) and temperature (50 C or 100 C). In the second experiment, efficiency of extraction was determined using 1, 2, or 3 sequential static cycles at 50 C with methanol. These experiments were repeated six times. Additional experiments were conducted to determine the limit of quantitation and validate the method.

In all experiments, extraction cell pressure was maintained at 10.3 MPa. This pressure was chosen because it is near the center of the instrument's pressure range and has been used in previous experiments (*87*). Multiple pressures were not investigated, as the primary function of pressure is to keep the solvent in the liquid state at high temperatures (*92*). Before the solvent was added, the cell was preheated for 2 min. After the solvent was introduced, the cell was heated until thermal equilibrium was reached, which required 5 min. The static cycle was 5 min., during which time the cell contents were held at the desired temperature and pressure. After that time, the cell was flushed with fresh solvent equal to 60% of the cell volume, which was purged from the cell by a stream of N_2 gas for 60 s and expelled into the collection vial.

Limit of quantitation determination and method validation. The estimated limit of quantitation (LOQ) was determined separately for each soil because differences due to the organic components of the soil were expected. Limits of quantitation were determined by calculating the fluometuron concentration in soil that resulted in a detector response that was 10 times the background noise (*93*). This concentration was

0.043 µg fluometuron/g soil for field soil and 0.124 µg fluometuron/g soil for pasture soil. These limits were then tested experimentally by applying Cotoran at concentrations representing 0.5, 1, 2, and 10 times the calculated LOQ of fluometuron. Each treatment was replicated three times.

Ten grams of soil were mixed with 2 g washed silica sand and treated with Cotoran in methanol at concentrations of 8.2, 4.1, 2.04, 1.02, and 0.51 µg fluometuron/g soil. Herbicide-treated soil was thoroughly mixed and equilibrated for 24 hours prior to extraction. Samples were extracted with methanol at 50 C during three 5-min. static cycles. The experiment was replicated six times.

Chromatographic conditions. Samples were analyzed by liquid chromatography/ photodiode array (Waters Inc., Milford, MA) using a Symmetry Shield RP8 3.5 µm C8 2.1x150 mm column. The mobile phase was 50:50 acetonitrile:water, the sample injection volume was 10 µL, and the flow rate was 0.2 mL min^{-1}. Fluometuron was detected at 243 nm.

Statistical analyses. The experimental design in all studies was a randomized complete block. Data were subjected to analysis of variance using the mixed model in SAS (SAS Institute, Cary, NC) to evaluate the significance of any interactions and determine treatment means. The summary procedure in SAS was used to calculate the standard deviation of means.

RESULTS AND DISCUSSION

Extraction conditions. Recovery from pasture soil was greater than from field soil, with recoveries of 82% (RSD 9.0%) and 72% (17.4%), respectively. These results were not expected, as the presence of more organic matter in pasture soils was expected to cause enhanced binding of the pesticide and therefore reduced recovery (*94*). No fluometuron was recovered from non-fortified samples of either soil (**Figure 14**). There were no statistical interactions between soil and temperature, solvent, or number of static cycles.

When fluometuron was extracted at 100 C, the RSD was greater relative to fluometuron extraction at 50 C (**Table 12**). In addition, the higher temperature increased extraction of potentially interfering soil constituents. Therefore, 50 C was chosen as the desired temperature for extraction. The lower temperature also reduced the potential effects of thermal instability, which is a characteristic of fluometuron (*95*).

There was no difference between using methanol and acetonitrile when the main affect of solvent was considered (**Table 12**). When the combined effects of temperature and solvent were considered, there were no differences in fluometuron recovery by acetonitrile and methanol when both were used at 50 C (**Table 13**). Therefore, methanol was chosen as the solvent. This is consistent with previous reports (*96*) and indicates the ease with which shake flask methods can be adapted for ASE.

Repeating the static cycle three times yielded 78% recovery of fluometuron (RSD 8.1%). One or two repetitions yielded 72% (RSD 9.4%) and 76% (RSD 12.4%) recovery, respectively. Repeated static cycles increased extraction efficiency by

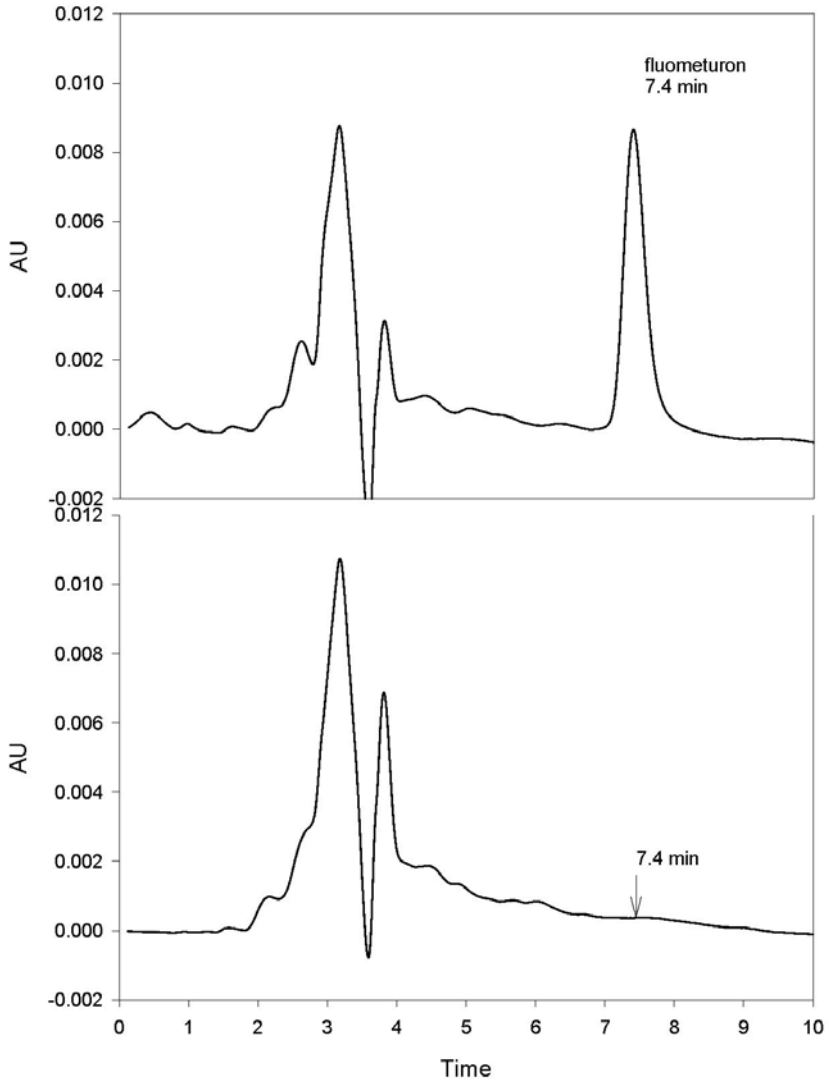

Figure 14. Typical chromatograms of extracts from pasture soil treated with 4.1 μg fluometuron g soil $^{-1}$ (top) and non-treated pasture soil (bottom). Extraction conditions were 1 static cycle at 50 C with methanol.

Table 12. Fluometuron recovery from pasture and field soil as influenced by main effect of solvent or temperature.

Extraction variable	Condition tested	Recovery	RSD
		%	%
solvent	methanol	74	17.2
	acetonitrile	79	13.9
temperature	50 C	78	14.5
	100 C	76	17.0

Table 13. Influence of solvent and temperature interaction on fluometuron recovery from field and pasture soil.

Solvent	Temperature C	Recovery %	RSD %
methanol	50	76	10.9
	100	77	20.9
acetonitrile	50	83	15.5
	100	79	13.3

increasing soil contact with fresh solvent. Increases in efficiency may also be obtained by increasing the static time during a single cycle.

Limit of quantitation and method validation. The LOQ was determined separately for the two soils, as background noise was greater in samples generated from pasture soils than field soils. Even though the calculated LOQ is often reported, this may not be accurate for a given method or matrix (*97*). Fluometuron concentrations of 0.043 and 0.022 μg/g resulted in recoveries greater than 300% and RSDs greater than 100% (**Table 14**). However, when fluometuron was applied at 0.086 μg/g soil, the recovery was 133% and the RSD was 12.1%. Therefore, the LOQ for fluometuron in field soil was determined to be 0.086 μg/g soil, which was twice the calculated LOQ. In pasture soil, the calculated LOQ of 0.124 μg fluometuron/g soil was accurate.

When the optimized method parameters were evaluated over a range of fluometuron concentrations, fluometuron recovery ranged from 81% to 126%. In general, both recovery and variance increased as concentration decreased; however, as noted in the initial experiments, recovery from the field soil was greater than from pasture soil (**Table 15**). Extraction efficiency from the two soils was consistent for concentrations between 1.02 and 8.2 μg fluometuron/g soil, which is a range that is likely to be biologically relevant with regard to herbicide carry-over (*98*).

The soil concentrations suggested here may initially appear insufficiently low. However, previous work (*17, 52*) was presumably based on a furrow slice depth of 15 cm, rather than 2 - 5 cm, as used in this work. The concentrations used in this study were chosen to be more representative of a depth where fluometuron will be optimally

Table 14. Fluometuron recovery from field and pasture soils near the estimated limit of quantitation.

Soil	Fluometuron concentration ng/g soil[a]	Recovery %	RSD %
field	22	376	149
	43	314	149
	86	133	15.1
	430	104	42.8
pasture	62	147	8.7
	124	120	2.1
	248	106	0.28
	1239	115	9.9

[a]Calculated limit of quantitation was 43 and 124 ng fluometuron/g soil for field and pasture soil, respectively.

Table 15. Recovery of fluometuron applied at five rates following accelerated solvent extraction.

Soil	Fluometuron concentration ng/g soil[a]	Recovery %	RSD %
field	8.2	88	2.7
	4.1	90	2.1
	2.04	93	2.5
	1.02	104	4.3
	0.51	126	4.1
pasture	8.2	81	3.9
	4.1	82	3.1
	2.04	85	6.3
	1.02	97	4.6
	0.51	121	4.3

[a]Concentrations represent 2.0, 1.0, 0.5, 0.25, and 0.125 times the concentration of fluometuron present in the upper 5 cm of soil following a field application.

concentrated after activation by rainfall. When converted to the soil concentration in a 15-cm soil depth, the concentrations used here produced similar results.

These experiments indicated that ASE can successfully be used to extract fluometuron residues from silt loam soils with a range of organic matter contents. This alternative technique will allow more rapid and efficient studies of fluometuron residues in soil. For example, extracting fluometuron using shake-flask methods requires at least 16 to 24 h shaking with a solvent:soil ratio of 2:1 (v:w) (*17, 95*). Using the method described here, each sample was extracted in approximately 20 min. and the extractant from 10 g soil was dissolved in approximately 15 mL solvent. Additionally, these experiments have demonstrated that little modification is needed to adapt conventional methods for use in ASE.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The experiments described in this document addressed some of the potential issues associated with the current patterns of glyphosate use in glyphosate-tolerant cotton, particularly with regard to the soil microflora.

Soil microbial activity was altered when glyphosate was applied with fluometuron, aldicarb, or mefenoxam + PCNB relative to when these pesticides were applied without glyphosate; however, soil microbial activity following trifluralin application was similar with and without glyphosate. Nitrogen mineralization in soil was increased when glyphosate was applied, regardless of the presence of other pesticides. Additionally, glyphosate application resulted in an increase in soil microbial biomass C when no other pesticides were present; however, the C content of soil microbial biomass was similar when other pesticides were applied alone or with glyphosate. Soil microbial biomass N was not affected by any pesticide treatment.

The addition of glyphosate with fluometuron resulted in different responses from bulk soil and pure culture *in vitro*. When soil was treated with fluometuron and glyphosate, the rate of fluometuron degradation was enhanced relative to when soil was treated with fluometuron alone. This increase in degradation was associated with increased soil microbial activity. However, when *R. solani* was grown in the presence of fluometuron and glyphosate, both the rate of fluometuron degradation and fungal growth were decreased relative to fluometuron alone. The different responses noted for microbial

degradation of fluometuron when applied with glyphosate to soil and in pure culture demonstrate the complex nature of microbial interactions with regard to pesticide degradation.

There were few differences in glyphosate dissipation or soil microbial community composition when glyphosate was applied 1, 2, 3, or 4 times. However, $^{14}CO_2$ evolution and extractable ^{14}C-residues were reduced and changes were detected in the FAME profile of the soil microbial community when glyphosate was applied 5 times. These studies suggested that the changes in the dissipation and distribution of glyphosate following repeated applications of glyphosate may be associated with changes in the composition of the soil microbial community.

These finding may indicate a need for producers to reduce their reliance on the herbicide glyphosate in order to preserve the microbial characteristics of a given soil. Reduced glyphosate use will likely be accompanied by an increase in the use of soil applied herbicides. However, the efficacy of these soil applied herbicides may potentially be compromised when used in glyphosate-based weed management systems.

Finally, the ASE method developed was successfully used to extract fluometuron residues from both silt loam soils and *R. solani* biomass. The use of this method demonstrated the ease with which conventional extraction methods can be adapted for use in ASE.

As the prevalence of crops tolerant to glyphosate and other herbicides increases, it will be beneficial to continue to study the influence of pesticide interactions on the soil microbial communities. Further research could be focused on determining the effects of

glyphosate on the fate of additional pesticides and plant diseases. In addition, more research is necessary to determine what genetic alterations in the soil microflora are associated with the changes in the activity, function, and composition of the soil microbial community observed in these experiments.

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APPENDIX

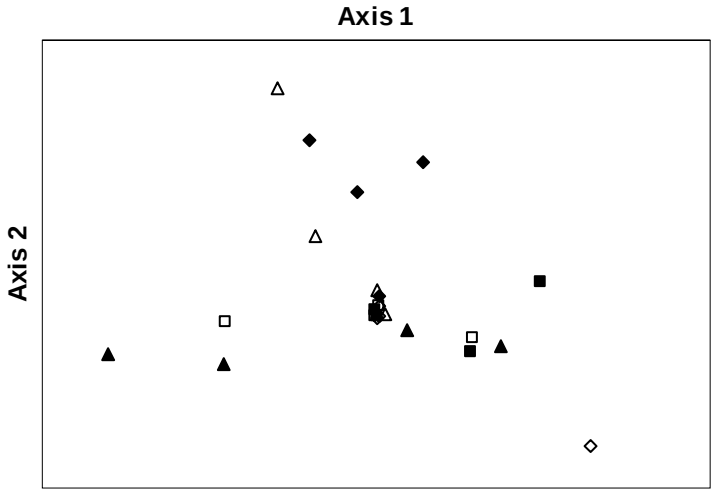

Figure A1. Nonmetric multidimensional scaling analysis of fatty acid methyl esters extracted from soil 3 days after 0 (◊), 1 (□), 2 (Δ), 3 (♦), 4 (■), and 5 (▲) glyphosate applications.

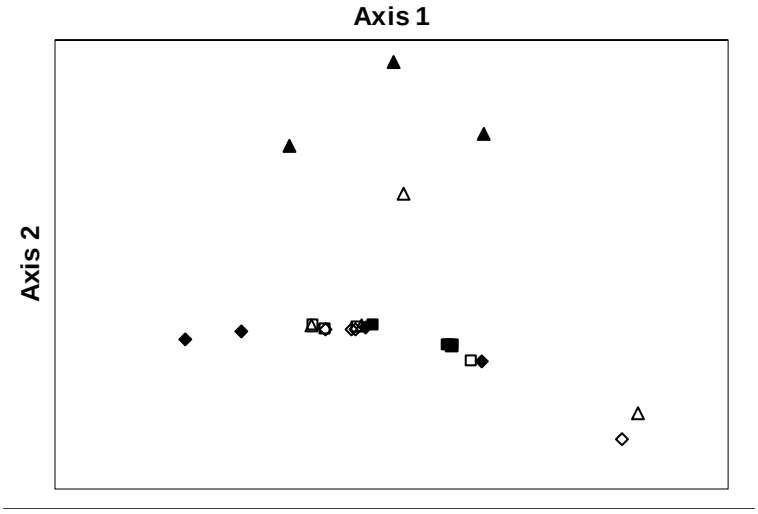

Figure A2. Nonmetric multidimensional scaling analysis of fatty acid methyl esters extracted from soil 7 days after 0 (◊), 1 (□), 2 (Δ), 3 (♦), 4 (■), and 5 (▲) glyphosate applications.

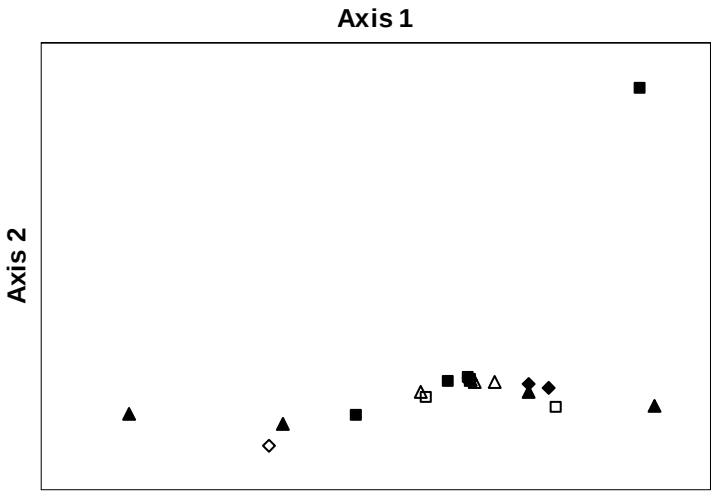

Figure A3. Nonmetric multidimensional scaling analysis of fatty acid methyl esters extracted from soil 14 days after 0 (◊), 1 (□), 2 (Δ), 3 (♦), 4 (■), and 5 (▲) glyphosate applications.

Table A1. Fatty acid methyl esters differing in concentration 7, and 14 days after application (DA) of glyphosate[a].

	15:0 3OH		17:0 cyclo		18:1 ω5C		19:1 ω8 al		20:0	
	7 DA	14 DA	7 DA	14 DA	7 DA	14 DA	7 DA	14 DA	7 DA	14 DA
Applications	% of response									
1	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
2	0.02 ab	0.0 a	0.02 ab	0.0 a	0.01 ab	0.01 a	0.02 ab	0.0 a	0.0 a	0.0 a
3	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
4	0.0 a	0.01 ab	0.0 a	0.01 ab	0.0 a	0.0 ab	0.0 a	0.02 a	0.0 a	0.02 ab
5	0.05 b	0.05 b	0.04 b	0.04 a	0.03 b	0.04 b	0.06 b	0.05 a	0.06 b	0.06 b
Pr>F	0.0166	0.0112	0.0277	0.0147	0.0207	0.0093	0.0230	0.0388	0.0014	0.0106

[a]Means within a column followed by similar lowercase letter are not significantly different according to Tukey's test ($P \leq 0.05$).

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